THE REGENERATIVE AND IN VITRO MULTIPLICATION CAPACITY OF APICAL TISSUE OF ROSMARINUS OFFICINALIS L, ON MEDIUMS WITH LOW HORMONES CONTENT

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Abstract

A plant with phyto-pharmaceutical and therapeutic properties, Rosmarinusofficinalis L., of Mediterranean origin, is one of the ameliorated and perennial species in our country's climate, but it faces difficulty taking root in classical conditions, which is why it can benefit from the advantages of "in vitro" multiplication. The plant is known for its active principles due to volatile oils, tannin, saponosides and nicotinic acid, all of which can be extracted from Rosmarinifolium, but also for its therapeutic abilities, such as being a bile diuretic, a spasm and unpleasant state calming agent, a muscle pain and a rheumatism analgesic etc. With the passage of time, plant biotechnologies have proven to be a genuine success and are applied to the purpose of multiplying various species, be they culture, ornamental, medicinal or aromatic plants, and the technique has been expanded to include amelioration activities. Our experiment began in the month of May and aimed to study the multiplication ability of Rosmarinusofficinalis L. apical tissue on environments with low phytohormone additions, in the following variants: Mt = MS; V_1= Mt + 0.2 mg/l BA + 0.3 mg/l AIA (for regeneration and multiplication); V_2 = Mt + 8.0 mg/l 2,4D (for callus initiation). Observations were made after 20 and 45 days respectively. After 20 days, the evolution is weak on all variants; it is only after 45 days that in vitro regeneration occurs on the control sample (Mt) and multiplication occurs on the low hormonal composition sample (V_1), while callus generation can be observed on the V_2 sample (which required transfer to fresh medium after approximately 3 days from differentiation). We recommend an in vitro culture of the rosemary apex, on low hormonal balance medium (0. 2mg/l BA+0.3 mg/lAIA), for 45 days, in order to achieve advantageous multiplication on mediums with low doses of phytohormones. In order to regenerate callus, be it friable or regenerative, we recommend high doses of auxine (8-10mg/l 2,4D) and cytokinin (cca. 5.0 mg/l up to la 8 mg/l BA).

Keywords: *Rosmarinusofficinalis L.in vitro, ex vitro*, regeneration pace, multiplication, friable and regenerative callus differentiation, hormonal balance equilibrium, BA, AIA, 2,4D, necrosis, stagnation.

INTRODUCTION

Rosmarinus officinalis L. (fam. Lamiaceae syn. Labiatae) is a species well known since houndreds of years mainly for the parfumed oil (Munteanu L. S. et al, 2007). It has the popular name "mirtin" or "rojmalin" in Romanian. It is cultivated for Herbarosmarini-the plant as a whole(Bojorand Alexan, 1983) and also for the leaves Foliumrosmarini, part of the plant that ensures the valuable oil production, oil having a beautiful parfume and great quality(Paun E., et all 1988).

The plants is well known due to phyto-pharmaceutical proprieties and also for the contents of the volatile rosemary oil, rich in rosmarinic acid, flavones, hydrocarbon, alcool, vitamins, etc (Bojorand Alexan, 1983), being

used for numerous therapies as it has certain bacteriostatic and coleretic action (Paun et all, 1986). In ancient, empirical medicine the rosemary oil has been used as a vermifug or in gastrointestinal disease, as analgetic, in rheumatism and nevralgia (Munteanu L. S. et al, 2007). The leafy shoots are used as aromatic addition for meat preparation, as a very appreciated spice (Munteanu L. S. et al, 2008), as well as in cosmetics and perfumery.

The ecology of this species emphasizes its Mediterranean origin as it behaves in original areas as an evergreen shrub (Pârvu C., 2004). In our ecological conditions, rosemary behaves like a perennial plant not a shrub (Flora RPR, 1952-1974). Rosemary is a rather pretentious plant regarding the climatic conditions. It loves the light and warmth and has a real sensibility towards weather conditions, even at -1oC and -2oC temperature (Munteanu L. S., et al, 2001).

Rosemary also has high humidity demands. As far as the soil is concerned, rosemary prefers the light, permeable soil (easily getting warm), south oriented and away from air currents. The above mentioned conditions are met in the South-Eastern part of Romania (Constanta county) where this plant is cultivated (Pârvu C., 2004). The rosemary culture last for more than one year but also needs crop rotation. As rosemary does not have great demands regarding soil fertilization, the culture can be brought back on the same soil after 7-8 years (Roman, et all, 2012). The administration of natural fertilization is recommended (cca. 25 t/ha manure) and even the administration of chemical fertilizers is accepted in small amounts (Munteanu L. S., et al, 2008). The success of the culture can be ensured by the care works applied to it during the vegetative period (Roman, et all, 2012). Lately, the culture of rosemary is widely used in landscape gardening as ornamental species, as it is more and more appreciated (Botanica-Encyclopedie de botanique et d'horticulture, 1997). Rosemary is appreciated for the colour and perfume of the flowers, properties well kept in time (Encyclopedie universelle des 15000 de plantes, 1999). Mainly this plant is appreciated, valued and expanded in culture due to its active principles: volatile oil, tannin, saponozides, etc. It also has therapeutic properties (Nazadt L., coord., 1993) and due to this fact, applied biotechnology in the case of rosemary showed beneficial as used for in vitro propagation (to expand and improve its cultivation) and also to obtain bio-mass with phyto-terapeutical properties (Păun E., et al, 1986, 1988).

Under the climate conditions from Romania, Rosmarinus officinalis L. does not produce seeds, thus its propagation takes place through seedlings and cuttings. This is a species with rather hard rooting and in vitro propagation is very beneficial (Cachita C. D., 1987). Getting the seedlings (after purchasing the proper seeds with a good germination capacity) need cultivating in greenhouses and warm shelters, in order to obtain the plants

until May for having enough time for the whole cultivating cycle (Munteanu L. S., et al, 2001). Under the climate conditions of our country it is not a good economic solution to obtain seedlings because they need special conditions of warmth, humidity, light (warm shelters and greenhouses) during the spring period (Munteanu L. S., et al, 2003). The saving solution is in vitro propagation on the purpose to obtain a valuable quantity of vegetative material (Laslo V., 2011). Rosemary, alike all fragrant and medical plants are studied for obtaining in vitro generated calluses bio-mass, a superior quality tissue (Cachita C. D., et al, 2004).

HerbaRosmarinii and FoliumRosmarinii is obtained during the plant harvesting and depends on culture conditions and on the harvesting momentum (dry, sunny) and ensure delivery of a very valuable bio-mass (Paun E., et al, 1988).

In our experiment, in vitro propagation of Rosmarinus Officinalis L. has been planned in order to obtain a large number of plants at a very law cost by application of a lucrative technology (Bajaj, 1986), by using simple mediums, basic mediums or phyto-hormones supplemented mediums :0,2 mg/l up to 0,3 mg/l (Cachita C. D. et al, 2009). In addition to simple mediums with low concentration of hormones, in order to obtain low cost in vitro cultures, we know that we can replace partially and totally the hormones with natural extracts (Butiuc-Keul A, Zăpârţan, M., 1996).

These kind of economic technologies are successfully used to obtain seedlings and are introduced to culture (Agud E. et al, 2010, Agud E., 2011). Vegetative bio-technologies have proven a real success, applied to improvement of diverse ornamental species cultivation (Laslo V., 2013) and are extended to some other medical and phyto-pharmaceutical species (Zăpârţan M., Deliu C, 2001).

Besides the improvement of culture, in vitro propagation technology aims to increase the conservation possibilities of genetic resources and of medical value plant species (Withers L. A., 1990) and also germplasm conservation (Engelman F., 1997).

This technology also initiates research for finding the most favorable method in order to in vitro conservation of certain plants, mostly of those threatened by extinction (Fay M. F., 1992). Spontaneous flora of our country knows the benefit of conservation through un-conventional methods of in vitro propagation (Laslo V. et al, 2011), with certain preoccupation regarding protected area of some regions for conservation of botanical elements from these areas that are threatened with extinction (Agud E., 2014).

MATERIAL AND METHODS

This experiment has been initiated in May. From a young but mature seedling an apex of 0.5 cm has been cut and it was in vitro inoculated. Apex has proved during our experiments a great and superior capacity for regeneration and propagation (Laslo V. et al, 2011). Apical tissue or meristem apical tissue has proved efficient for in vitro propagation of economical interest species and also for some potato species improved in our country (Agud E., et al, 2010). Plant in vitro propagation can be ensured from other types of tissues and explants. In some medical and fragrant species the knot, with hormonesin proper dose, can ensure an unimaginable capacity of propagation (Zăpârţan M., Deliu C, 2001). The experimental culture media have been simple, with small amount of phyto-hormones and have had the basic medium compounds MS (Murashige Skoog, 1962) with the following variants: Mt=MS; V1=Mt+0,2 mg/l BA+0,3mg/l AIA; V2=Mt+8,0 mg/l 2,4D. We conceived a formula with very small amounts of phyto-hormones (one auxine AIA and one cytokinine BA) in very small amounts for (V1), in order to stimulate regeneration and propagation, compared with control variant Mt (only with basic MS) and one variant was put up for calluses differentiation (V2).

RESULTS AND DISCUSSION

The observations have been made in two phases, after 20 and after 45 days and we followed the type of evolution (uniform or un-uniform) depending on the variant and it was expressed in % of regeneration, number of differentiated seedlings and the value of rooting system. The values of these parameters have been put in table 1, depending of the period of the observations.

After 20 days of culture, the apex of Rosmarinus officinalis L. has shown a uniform evolution, differentiated depending of the variant's composition. The apex on Mt stagnates after 20 days; on V_1, with small to moderate composition of phyto-hormones, it generates a satisfactory development, with two seedlings/apex, of cca. 1 cm in length, without roots, having a regeneration percent of 24% (table 1). On V_2, for calluses the differentiation is small, of θ 0.2-0.3 cm.

After 45 days of *in* vitro culture, the evolution of the *Rosmarinusofficinalis* L apex is conclusive, manifesting in accordance with the medium variant. On the control variant, with only Murashige-Skoog base medium (Mt), over 50% regeneration occurs, with the differentiation of a single plantlet of approximately 2.8 cm in length (thus not showing multiplication), and with only one fragile root.

Tabel 1

The *in vitro* evolution of *Rosmarinusofiicinalis* L apex (after 20 and 45 days respectively)

Days/ Var.	20 days			Observations	45 days			Observations
	Regen. (%)	No.pl/cm	No. Roots		Regen. (%)	No.pl/cm (average)	Nr. roots/cm (average)	
Mt	-	-	-	Stagnates	48	1 pl./2.8cm	0.5 cm	Satisfactory evolution
V_1	24	2 pl	-	Uniform evolution	90	10-12pl/ de 1.5-5.0cm	8roots. /2.5 cm	Very good evolution
V_2	callus	-	-	Callus. 0.2 - 0.3cmØ	Callus 78%	-	-	Callus mass of approx. 1 cmØ

The V_1 variant, with added hormones, albeit in low concentrations (0,2mg/lBA+),3mg/lAIA) determines a regenerative capacity of the apical tissue, which reaches a very good level of 90%, with the differentiation of 10-12 neo-plantlets/apex, of 1.5 – 5.0 cm in height. On the variant with an addition of 2,4D (V_2) in large concentrations (8mg/l), differentiation of callus mass occurs, of approximately 1.0cm Ø, olive-green in color, hard, with traces of necrosis; this tissue was transferred to fresh medium with two purposes in mind:

A. In order to stimulate regenerative embryogenetic callus differentiation: the transfer is performed on a medium with a medium dose of cytokinins (2,0-3,0mg/l) and a low dose of auxine (0,5mg/l);

B. In order to stimulate differentiation of friable callus, in order to obtain a quantity of biomass, either „*via callus",* or „*via cellular suspension*", through the transfer of the tissue on an environment with a high dose of 2,4D (cca, 8mg/l) but also in the presence of a large dose of cytokinine (cca. 5-8 mg/lBA).

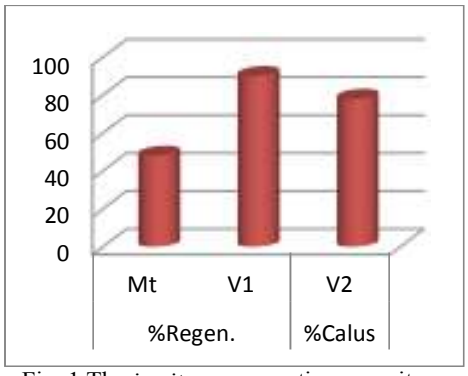

Fig. 1 The *in vitro* regenerative capacity of *Rosmarinusofficinalis* L apex tissue (% after 45 days)

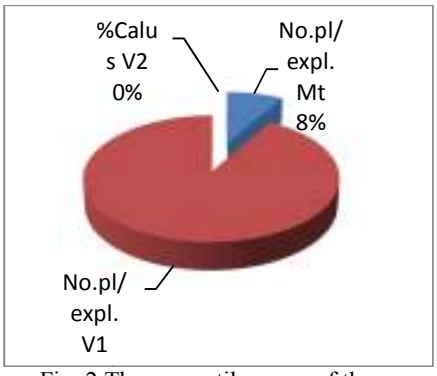

Fig. 2 The percentile mean of the number of neo-plantlets/explant (after 45 days)

The regenerative ability of the apex of *Rosmarinusofficinalis* L, after 45 days of *in vitro* culture on the experimental mediums (with a low input of phytohormones and low 2,4D content for the stimulation of callus formation) is presented in fig. 1. Observing the figure, we remark on the superior value of the parameter on medium with hormonal addition, as the regenerative capacity of the apical tissue of rosemary is over 90%, but we also highlight the fact that the base medium also condones regeneration capacity of over 45%. We can affirm the fact that the species responds favorably from a regenerative stand point to low and balanced doses of phytohormones, which is why we recommend the initiation of *in vitro* cultures on cost-efficient mediums, with a well-balanced hormonal ratio.

Regarding the *in vitro* multiplication, the *Rosmarinusofficinalis* L species exhibits a good percentage on base medium, and a very good percentage (over 90%) on the V_1 medium, with added hormones, values presented in figure 2, which highlights the percentile mean of the number of neo-plantlets differentiated from one rosemary apex. On the V_2 medium, enhanced with 2,4D, auxine involved in the differentiation of callus tissue to a percentage of 78% after 45 days of culture, there was no differentiation of *in vitro* plantlets. The differentiation of neo-plantlets from callus is stimulated by the presence of a cytokinine in a dosage of 2-3mg/l and of an auxine in a small dose of 0.5mg/l.

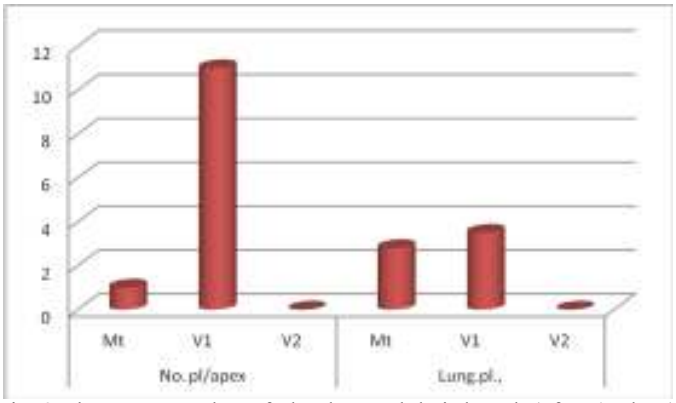

Fig. 3 The mean number of plantlets and their length (after 45 days)

The mean number of plants differentiated from a rosemary apex, and the length of these neo-plantlets, are presented in figure 3: on a medium with moderate doses of phytohormones (V_1) the apex can differentiate over 10 plantlets/apex, which grow to a length of over 2 centimeters, with a rich root system of approximately 8-10 roots/neo-plantlet, of approximately 2 – 2.5 cm in length, so the formation of completely organized plantlets can be observed. On the control medium, the apex differentiates approximately 1-2

plantlets which form fragile roots of approximately 0.5 centimeters in length (after 45 days of *in vitro* culture). The average of the values remarked upon is also presented in Table 1, with the comparative evolution of rosemary apical tissue after 20 and 45 days respectively of *in vitro* culture. The evolution of the apex is suggestively shown in photos 1, 2 and 4 (after 45 days of *in vitro* culture).

Photo 1 shows the evolution on base medium (Mt), on which we remark the lack of multiplication (only one plantlet was regenerated) and the lack of a root system. On the variant with phytohormones (Photo 2), evolution is obvious from the stand point of good multiplication and organization of neo-plantlets.

The evolution of callus tissue from rosemary apex (Photo 3) highlights olive-green tissue, with a diameter equal to the flask, but which exhibits traces of necrosis after 45 days, which is why we believe that after approximately 30 days of *in vitro* culture, it must pe transferred on fresh medium in order to achieve the sought-after goal: regeneration of plantlets or obtaining friable callus for cellular suspension and the eventual obtainment of biomass with determinable biochemical properties.

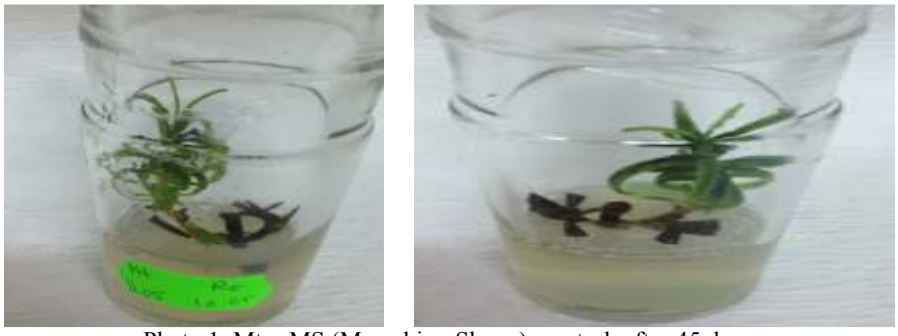

Photo 1. Mt = MS (Murashige-Skoog), control, after 45 days

Photo 2. V_1 = Mt + 0.2 mg/l BA + 0.3 mg/l AIA

Photo 3. V_2 = Mt + 8.0 mg/l 2,4D

CONCLUSIONS

- After 20 days of *in vitro* culture, the apex tissue of Rosmarinofficinalis L exhibited weak but uniform evolution, in relation to the composition of the variant mediums.
- Multiplication only occurs on the V_1 medium **(**Mt + 0.2 mg/l BA + 0.3 mg/l AIA), the variant with moderate to low phytohormone enhancement, exhibiting uniform and satisfactory evolution and generating 2 neo-plantlets/apex, of approximately 1 cm, without a root system, with a regeneration percentage of 24%.
- After 45 days of *in vitro* culturing, the evolution of the *Rosmarinofficinalis* L apex tissue is good, depending on the composition of the variant mediums.
- On the control medium (Mt), over 50% differentiation is observed, while a single plantlet is differentiated, without multiplication; on V_1 the regenerative capacity reaches 90%, which is very good, with differentiation of 10-12 neo-plantlets/apex, of approximately 1.5-5.0 cm in height; on V_2 an approximate diameter of 1.0cm Ø callus is generated, olive-green in color, hard, for which transfer is recommended.
- We recommend over 45 days of *in vitro* culture for the rosemary apex, on a medium with a low hormonal balance (0.2 mg/l BA+0.3mg/lAIA) in order to achieve multiplication in cost-effective conditions. Also, in order to regenerate friable or regenerative callus, we recommend high doses of auxine (8-10 mg/l2.4D) and cytokinine (5.0-8.0 mg/l BA)

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