IN VITRO REACTION OF THE MAJORANA HORTENSIS MOENCH SPECIES DEPENDING ON THE NATURE OF THE CYTOKININ AND THE EXPLANT

Agud Eliza Maria*, Laslo Vasile*, Zăpârţan Maria

*University of Oradea, Faculty of Environmental Protection, 26 Gen. Magheru, St., 410048 Oradea; Romania: eliza_agud@yahoo.com; vasilelaslor@yahoo.com

Abstract

The effect of various cytokinins, in combination with an auxin, was studied to determine the potential for in vitro regeneration and multiplication stimulation of the apex and the detached nodule in the Majorana hortensis Moench species, which is known for its richness in aromatic and phyto-pharmaceutical chemical compounds.The MS growth mediums used are: ***Mt*** *(control) = MS;* ***V_1*** *= Mt + 1.0 mg/l 2iP + 0.5 mg/l AIB;* ***V_2*** *= Mt + 1.0 mg/l K + 0.5 mg/l AIB; V_3 = Mt +1.0 mg/l BA + 0.5 mg/l AIB;* ***V_4*** *= Mt + 1.0mg/l TDZ + 0.5mg/l AIB;* ***V_5*** *= Mt + 6.0 mg/l 2,4D + 0.5mg/l BA. The study followed regeneration capacity (%), marjoram apex and nodule multiplication (differentiation from neoplant/explant) in the presence of the four cytokinins, but also the growth of callus on V_5, noting the complete organization of the new plants depending on the nature of the cytokinin. After 45 days of in vitro culture, good and uniform evolution was found on the BA (V_3) and 2iP (V_1) mediums:90% apex regeneration (on V_3) and 75% (on V_1), with 4-5 neoplants/apex, well rooted. In vitro nodule reaction on the same two mediums was good, but inferior to apex: on V_3 there was 80% nodule regeneration with cca. 3 neoplants 3 cm tall, rooted; on V_1 regeneration was at 60% with the same number of neoplnts/nodule. Weak evolution of explants was found on V_4, with TDZ, (50% of explants showing necrosis), and no in vitro reaction on V_5: we believe that the small dosage of BA is incapable of generating callus tissue differentiation. Following these observations, we recommend: for callus production, increasing the dosage of BA or using other cytokinins alongside it (cca. 6 mg/l) in combination with 2,4D (6mg/l); for multiplication, we recommend BA and 2iP (1mg/l) with a small dosage of auxin (0.5mg/l) and removing TDZ, which inhibits in vitro explant evolution, a reaction we have so far only encountered in marjoram.*

Key words: *Majorana hortensis Moench. in vitro multiplication, ex vitro*, *nod*, *apex*, regeneration (%), organogenesis, cytokinins: 2iP, BA, K, TDZ, auxins: AIB, 2,4D

INTRODUCTION

The use of medicinal and spice plants in the Carpathian-Danube-Black Sea region has a particular history and tradition, and academician T. Săvulescu (1924) believed that "folk botany was born in this region at the same time as the people, it evolved with it, and in it we can observe the past, the history, the crafts, the joys and sorrows of the people". Even more so, folk medicine, in its richness, differentiates between plants associated with mythological figures from the ones with healing and magical properties, and their harvest, preservation and use follows a complex ritual (Alexan M. et al., 1992).

Majorana hortensis Moench. (Lamiaceae fam.), synonymous with *Origanum majorana* L, or marjoram in common language, has been known

and appreciated since Classical Antiquity for its essential oils, bitter substances, tannin and other chemical compounds Bojor and Alexan, 1983). *Herba Majoranae* is used fresh or dried, the concentration of compunds and essential oils reaching up to 0.7-3.5% (Păun E.et al., 1986). It is known to also possess phyto-pharmaceutical properties, used for stomach troubles or an antiseptic etc. (Bojor O., Alexan M., 1983), as infusion for stimulating digestion, countering stomach aches, insomnias and calming nerves (Muntean et all., 2007). The species is also well known and used as a spice, in the kitchen, in the food and cosmetics industries, especially in the manufacturing of perfumes (Nazadt L., coord., 1993).

From an ecological standpoint *Majorana hortensis* Moench (marjoram) originates from the southern areas of the Mediteranean Sea, where it grows perennially (Pârvu C., 2004), having its own ecological properties. In our country, as in all areas in this part of Europe, it behaves as an annual plant with certain climate requirements: it prefers warm weather, light and humidity, it develops on warm, light, fertile, loose soils, with a neutral pH and containing calcium (Muntean L. S., et al. 2008). Regions in our country that are favorable for the cultivation of marjoram are the West of the country and Neamt county in Moldavia, and the South-West of the Romanian Plain (Flora RPR, 1952 – 1974), where it requires a specific crop technology that takes into account aspects pertaining to crop rotation, fertilizer use depending on the soil available, a particular sowing and seed production technique, as well as maintenance work typical for the vegetation period Roman et al., 2012).

Classic propagation of the *Majorana hortensis* Moench is sowing directly on the field, even seedling in colder areas. Sowing is done in April using the universal technique, the disadvantage to the marjoram seed being that it is small and frequently shriveled, it is mixed with an inert material (sand, sawdust), and a precursory plant, in this case, salad (Munteanu L. S., 2001). Propagation through seedling is harder to achieve due to needing special spaces (greenhouses and warm or semi-warm hotbeds), with a relatively small number per square meter, cca. 30 plants/sqm (Munteanu L. S., et al., 2003). This species does not respond well to vegetative propagation and a high percentage of the seeds obtained are shriveled, therefore *in vitro* is the ideal solution in order to create valuable material, both in quantity and quality (Cachiţă C. D., 1987).

The technology applied to mature plants in open spaces, in order to obtain the end product *Herna Majoranae,* ensures certain conditions at the moment of harvest, and depends on the optimal time in the crop development, on the start of blooming, as well as on the weather conditions at the time of harvest (clear, no dew or humidity). This is followed by the drying of the naturally or artificially harvested product at a temperature of

30-25ºC, then by the correct packaging and depositing up until delivery of the end product (Păun E., et al., 1988).

Treaties of vegetal biotechnology aim to prove the efficiency of some *in vitro* multiplication methods and of some breeding methods for plants that hold an economic, botanical or ornamental value (Cachiţă C. D., et al., 2004). Recent studies present the applications of vegetal biotechnology (Laslo V., 2013), among them its role in conserving some species of spontaneous flora in danger of extinction (Laslo V., et al. 2011; Agud E., 2014), and also conserving some indigenous fruit-growing species for future improvement (Laslo V., 2006). Research in the field has proliferated, in its efforts to find the best method to apply to the conservation of genetic resources (Fay M. F., 1992; Engelman F., 1997).

MATERIAL AND METHODS

In vitro culture of the *Majorana hortensis* Moench species was initially designed in order to obtain a great number of plants identical to the parent plant, to adapt and expand them in crops, but later also proved efficient in conserving varieties and breeding lines of indigenous plants (Bajaj, 1986).The role of cytokinins in the processes of *in vitro* multiplication and regeneration has been studied on many plants (with an economic, ornamental, medicinal, aromatic etc. value) and all types of cytokinins were found to be involved in these processes, depending on other occurring factors: sampling time, age and nature of the explant tissue, nature of the species, hormonal balance used etc. (Deliu C., et al, 1993; Butiuc-Keul, et al., 2003).

The experiment started in April, from young plants germinated *ex vitro*, as this is the optimal time from the standpoint of maturation of the tissue donating the explant – the apex – for inoculation and the start of the experiment, ensuring a certain degree of multiplication. The *apical meristem* (apex) is considered the most efficient explant in *in vitro* micro-multiplication of some economically valuable species, like some varieties of potato (Agud E., et al. 2011). *In vitro* propagation in plants can be ensured by many other types of explants, as in the case of the node explant taken from the medicinal herb *Mentha piperita,* with remarkable results in micro-propagation (LasloV., et al., 2011). The role of some hormones and the implications of the hormonal balance in the medium were proven to induce *in vitro* organogenesis and to stimulate regeneration and multiplication capacity in some ornamental species (Zăpârţan M., 1996), as well as in some spontaneous flora species, with the purpose of conserving some rare botanical elements (Agud E., 2014).

In establishing the composition of the mediums we took account of some of our own previous results in stimulating multiplication in other species. The baseline growth medium used (Murashige and Skoog, 1962), was added the cytokinins available to us: 2iP, BA, K şi TDZ (Tidiazuron) with the same moderate concentration (1mg/l) and a low dosage of auxin (0,5mg/l), resulting in the following variants: **Mt** (control) = MS; **V_1** = Mt + 1.0 mg/l 2iP + 0.5 mg/l AIB; **V_2** = Mt + 1.0 mg/l K + 0.5 mg/l AIB; V_3 = Mt +1.0 mg/l BA + 0.5 mg/l AIB; **V_4** = Mt + 1.0mg/l TDZ + 0.5mg/l AIB; **V_5** = Mt + 6.0 mg/l 2,4D + 0.5mg/l BA. *In vitro* regeneration and multiplication of species with a high phyto-pharamaceutical value, such as *Arnica montana* L (Zăpârţan M. et al, 1994; Zăpârţan M, 1996) and *Ginkgo biloba* L, is aided and stimulated by the presence of cytokinins in a moderate concentration (Deliu C., et al, 1994).

RESULTS AND DISCUSSION

Observations were done cca. 45 days (6-7 weeks) after the inoculation of explants from the apex and node *in vitro*: the evolution of marjoram explants, expressed in median values, is shown in Table 1. The reactions of the tissues concerning *in vitro* plant regeneration were similar, yet the apical tissue showed superior reaction, manifested especially through a uniform evolution on all growth mediums. If on the MS control medium (Mt) the apex forms 1-2 seedlings/apex, 6-7 cm tall, evolution on all other mediums is superior.

Table 1

Regeneration and micro-propagation capacity (differentiation from neo-plants) in marjoram depending on the nature of the cytokinin and the of the explant (after 45 days)

Var.	APEX			NOD			Observations
	No. of pl./ l(cm)	No. of roots/ l(cm)	% Reg.	No. of pl. /l(cm)	No. of roots/ l(cm)	% Reg.	
Mt	2 pl./ 7cm	2 roots / 0.3cm	65	2pl./ 6cm	2 roots/ 0.5cm	40	Good evolution, no multiplication
V_1	4pl./ 2cm	4 roots / 10cm	75	2pl./ 1cm	2 roots / 0.5cm	60	Uniform evolution
V_2	2pl./ 1cm	3 roots / 2-3cm	50	2pl./ 1cm	3 roots / 0.3cm	20	50% evolution 50% stagnation
V_3	5pl./ 2cm	5 roots / 8-12cm	90	3pl./ 3cm	2 roots / 0.3cm	80	Uniform evolution
V_4	0	0	0	-	-	-	Slow evolution. 50% stagnation
V_5	0	0	0	-	-	-	80% necrosis 20% stagnation

It is well known that, in other species, on growth mediums with no hormones regeneration results in the differentiation of 1-2 tall seedlings, on this simple medium, and even on medium MS½ (with quantity of macro and microelements halved) stem internode is stimulated to elongate, which favors another operation, that of *in vitro* mini-cutting, to obtain increased productivity of *in vitro* plant generation (Cachiţă C. D., 2006).

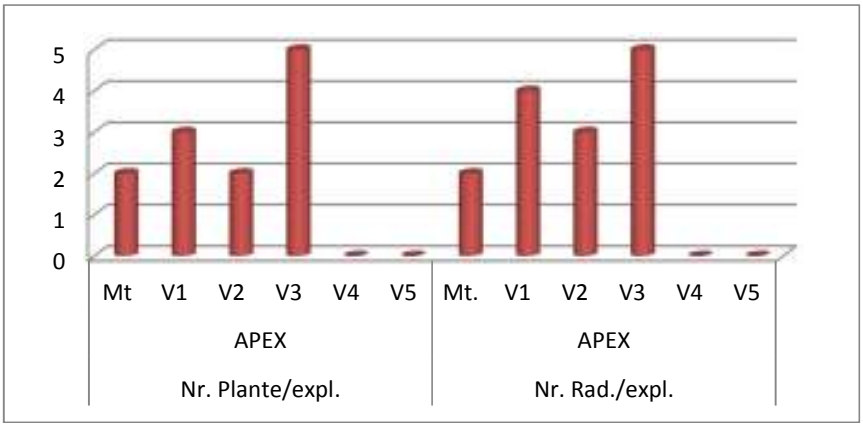

Fig. 1. Differentiation of seedlings and roots from the apical meristem in *Majorana hortensis*

On variants with phyto-hormones (various cytikinins + a single type of auxin) the presence of 2iP (V_1) and of BA (V_3) in the MS medium determined the best evolution, causing regeneration of 4-5 seedlings cca. 2-2.5 cm long, with 4-5 roots/seedling (10-12 cm long). The regeneration capacity of apical tissue of marjoram is presented in Figure 1. As can be seen, variants with 2iP and with BA (V_1, V_3) are superior to the one containing kinetin (V_2), on which evolution is similar to that on the control growth medium (Mt), while the variant having TDZ (V_4) resulted in slow evolution and stagnation in 50% of explants. The variant conceived in order to differentiate callus (V_5= Mt + 6mg/l 2,4D + 0,05mg/l BA) resulted in the apical tissue almost entirely afflicted by necrosis, in our opinion due to the very small dosage of cytokinin being incapable of stimulating the differentiation of the primary callus tissue, as it is known that to induce callus the initial dosage has to be higher, subsequently, depending on the type of callus desired, the dosages of 2,4D and cytokinin are lowered and balanced (Deliu C., et al. 1994). The rate of apical tissue regeneration in *Majorana hortensis* L reaches 90% on the BA (V_3) medium and 70% on the 2iP (V_1) medium, the latter being close to the values registered on the control medium (Mt) – cca. 65-67% (Figure 2). As the figure shows, we can state that the Murashige – Skoog 1962 (Mt), through its base composition

(micro and macro FeDTA elements, vitamins etc.) can favor a high or very high rate of *in vitro* tissue regeneration, depending on the explant and the species involved (Cachiţă C. D., 1987).

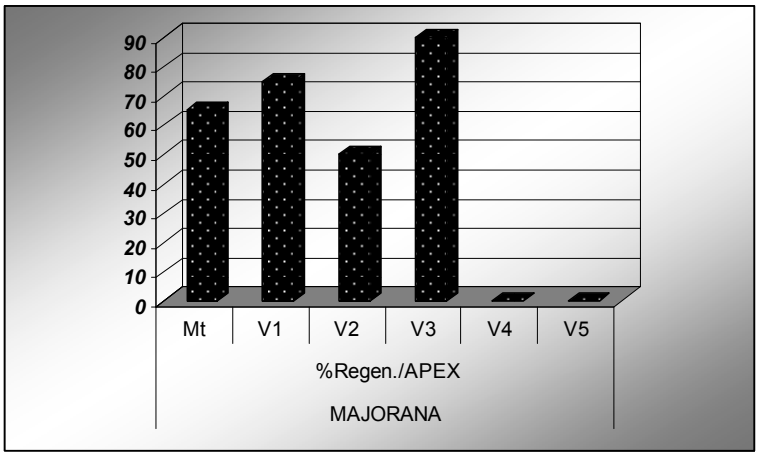

Fig. 2. In vitro regeneration percentage of the apical meristem of marjoram on various growth mediums with different types of cytokinins

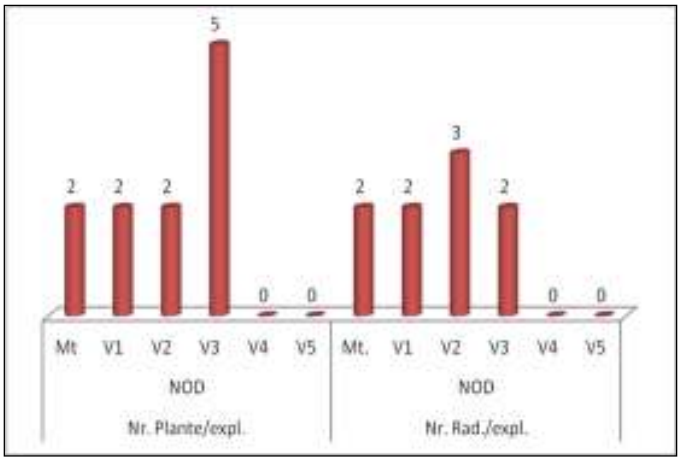

Fig. 3 Differentiation of seedling and roots from nodal tissue in Majorana hortensis

The node explant, after the same duration of *in vitro* culture (45 days) has a similar evolution to that of the apex, except median values on the parameter followed were somewhat lower. The number of seedlings/node on medium V_3 (with BA) is, in the case of this explant also, the highest, with a mean of 3-4 seedlings/node, cca.3cm tall with 2 short roots. On the other variants containing phyto-hormones regeneration yields cca. 2 new seedlings/node cca.1 cm tall; the control medium Mt generates the same

number of seedlings, but the latter are much taller, cca. 7 cm tall, once again showing the effect of the basic Murashige-Skoog medium (no phyto-hormones) on the elongation of internodes of the explant. Figure 3 below shows the differentiation of seedlings from marjoram nodes in direct correlation with root differentiation.

The relatively small number of differentiated roots in both marjoram explants (apex and node) is due to the low concentration of auxin (0.5 mg/lAIB), although the ß indolyl butyric acid is known to be most efficient auxin in stimulating rich, healthy root system differentiation (Cachiţă C. D. et al., 2004).

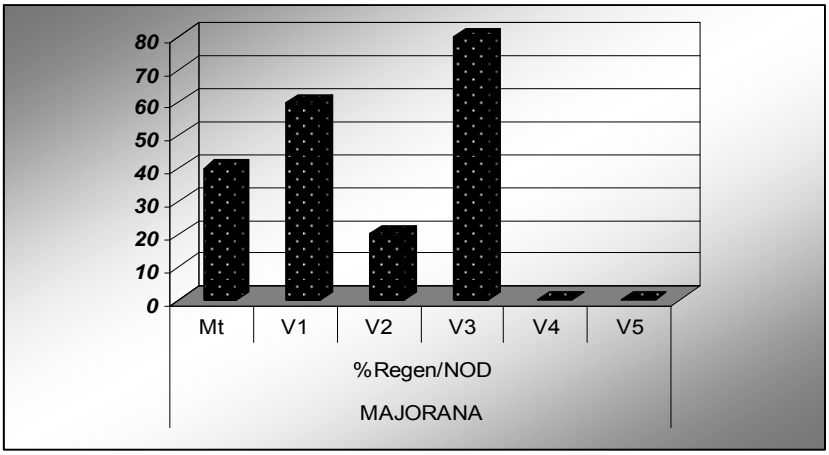

Fig. 4. Regenerative capacity (%) of *node* of Marjoram bred *in vitro*
On mediums with various types of cytokinins

The percentage of plant regeneration form nodal tissue reaches its highest value in the presence of BA (V_3) – 80% (10% lower than the regeneration rate obtained from the apex), but even in the presence of 2-isopentyl adenine – 2iP (on V_1) the rate still reaches a good value of 60%. It is to be noted that on both variants, the node evolution is uniform, as in the case of apical tissue. The control (Mt) yields 40% node regeneration, while in the presence of kinetin (V_2) the rate only reaches 20%. The *in vitro* regeneration rate of marjoram nodal tissue on the Murashige-Skoog growth medium, with various cytokinins added with the same balanced concentration (1 mg/l) and a small dosage auxin (0.5 mg/l) is presented in the graphic in Figure 4, highlighting once again the effect of the nature of the cytokinin and that of the tissue used upon *in vitro* regeneration capacity.

CONCLUSIONS

The evolution of apical and nodal tissue of *Majorana hortensis* Moench on growth mediums with different cytokinins with the same concentration and with a small dosage of auxin (0.5 mg/lAIB) is similar, with differences depending on the nature of the cytokinin and that of the tissue.

1. The best results for the *apex* were found on mediums containing BA (V_3) and 2iP (V_1), having a uniform evolution on all explants.

2. Multiplication in *Majorana hortensis* takes place in the presence of benzyl aminopurine (BA) and of 2-isopentyl adenine (2iP), reaching 90 and 70%, respectively, of apical explant: superior root system formation was recorded on the same variants, with a median of cca. 4-5 roots/explant cca. 10-12 cm long.

3. Kinetin in growth medium for marjoram leads to a reaction similar to the one on MS (Mt); the presence of Thidiazuron (TDZ) in the medium has proven inefficient for this species

4. The nodal explant has a similar evolution, but yield inferior values: in the presence of BA regeneration reaches 80%, and cca. 3 seedlings are obtained per node, cca 3 cm tall with small roots (0.3-0.5 cm)

For obtaining callus, increasing the dosage of BA or adding other cytokinins (2iP, K), with concentration of up to 6 mg/l, in combination with the 2.4D auxin – 6 mg/l. We also recommend for the multiplication of the species marjoram the cytokinins BA and 2iP, 1 mg.l each with a small dosage of auxin (0.5mg/lAIB) and discarding the cytokinin Tidiazuron (TDZ), which inhibits the *in vitro* evolution of the apical meristem and nodule tissue in marjoram.

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