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Micropropagation and Acclimatization of Common Oregano (*Origanum vulgare* L. Subsp. *vulgare*) by Shoot Tip Culture

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Abstract

Origanum vulgare L. is a commercially valued species with remarkable biological properties. It is subject to over-exploitation practices that seriously threaten its sustainability for future generations. Thus, micropropagation serves as a tool for the protection and domestication of this species. In this study, we established an *in vitro* vegetative propagation protocol for *Origanum vulgare*. This is done through the axillary bud technique by carrying out various tests. Six culture media (MS, MSm, $N_{30}K$, SD, SH and B5) were tested. Therefore, SD was chosen for the following experiments. Seven cytokinins (adenine (Ad), N6-(2-isopentenyl) (2ip), zeatin (Zeat), kinetin (Kin), benzyladenine (BAP), 1,3-diphenylurea (DPU) and thidiazuron (TDZ) at 5 concentrations (0.44, 1.33, 2.22, 3.11 and 4.44 µM/L) were evaluated. Thus, Kin at 3.11 µM allowed high regeneration of vitroplants, optimal elongation, total rooting of explants, maximum bud multiplication, and absence of hyperhydric explants. In fact, the integration of auxins (indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and 1-naphthaleneacetic acid (NAA)) into the culture medium and their combinations with 3.11 µM Kinetin contributed to the optimization of the root part. Thus, it was improved in particular in the case of 3.11 µM Kin and 6.27 µM IBA. Three polyamines (Putrescine, Spermidine and Spermine) at different concentrations (1.134, 3.402, 5.67, 7.938 and 11.34 µM/L) combined at 3.11 µM Kin and 6.27 µM IBA were tested. In fact, 1.304 µM putrescine was considered to be the most suitable for *in vitro* culture of explants, since it allowed optimal propagation of buds and roots,

also a high rate of regeneration and rhizogenesis. GA_3 at 1.15 µM combined with 3.11 µM Kin and 6.27 µM IBA permitted maximum bud multiplication. The acclimatization was carried out successfully using vitroplants showing good foliar and root development. Thus, three months after acclimatization, the seedlings were transferred into large pots under natural light and temperature conditions. Almost all acclimatized plants developed flowers in the first year between May and July.

Keywords

Auxins, Cytokinins, Gibberellic Acid, Macronutrients, Micropropagation, Polyamines, *Origanum vulgare*

1. Introduction

Origanum vulgare L. is an herbaceous plant, perennial, 30 - 80 cm long, branched and covered with hairs, a little reddish purple. It blooms from July to September [1] and is native to the Mediterranean region [2] [3] and grows on drained dry silt soils in warm regions and can tolerate low temperatures [4]. It is a hemicryptophyte plant whose organs for the bad season (drought or winter) are located at ground level. It is propagated from seeds or by asexual production [5].

Origanum vulgare is an important culinary herb and belongs among the most widely consumed spice plants in the world [6]. In addition, this plant has long been used in traditional medicine and the interest in the conception of new formulations in various fields is increasing. Several studies have shown that this plant contains a wide variety of secondary metabolites. The majority of them are phenolic compounds such as flavonoids, terpenoids, phenolic acids, alkaloids and also fatty acids among others [7] [8]. These main compounds are responsible for the biological activities which allow their use not only in the medical field, but also in food and cosmetic preparations [8] [9]. Several investigations have demonstrated several therapeutic properties: antimicrobial [10], antipasmodic [11], antiviral [12] and neuroprotective [13]. For these reasons, *Origanum vulgare* is one of the most commercially popular species with remarkable biological properties. As a result, its global marketing and consumption are constantly increasing [14]. In fact, the growing demand in the pharmaceutical, perfumery and cosmetics industries requires large-scale production. Currently, most of the plant material of *Origanum vulgare* is harvested from nature, which favors its disappearance in natural populations [15]. In addition, it is subject to overexploitation practices that seriously threaten the sustainability of its resources for future generations. The conservation of this species remains an important necessity for reducing its overexploitation.

Conventional propagation techniques have several disadvantages, namely large-scale propagation by seeds is subject to several barriers such as low germination, low viability, seed sterility, repeated vegetative propagation that causes

progressive yield loss, and finally, low rooting capacity of cuttings [16]. Also, the search for alternative pathways through the *in vitro* cultivation of these species is a promising biotechnological strategy for the conservation of the species and their large-scale production. This allows rapid mass propagation and maintenance of its durability.

In this study, nodes with two axillary buds of *Origanum vulgare* were used as explants. The objective was to establish an *in vitro* vegetative propagation protocol by carrying out various tests to optimize its development. This allows the needs of the farmer to be met and an alternative culture of *Origanum vulgare* to be introduced in northern Morocco.

2. Material and Methods

2.1. Plant Material

The explants used in this study were obtained from the apex of 3 to 4 cm of 4-week young plantlets of *Origanum vulgare* L. preserved in the Laboratory of Plant Biotechnology.

2.2. Effect of Mineral Nutrients

The mediums tested were MS (Murashige and Skoog, 1968) [17], SD (Shah and Dalal, 1978) [18], modified MS (MSm) (Badoc, 1982) [19], N30K (Margara, 1978) [20], B5 (Gamborg 1968) [21] and SH (Schenk and Hildebrandt, 1972) [22]. All of them were added with MS micronutrients and vitamins and 3% sucrose. The best macronutrients were served for all the following tests.

2.3. Effect of Cytokinin

Seven cytokinins: Adenine (Ad), (2-Isopentenyl)adenine (2ip), Zeatin (Zeat), Kinetin (Kin), 6-benzylaminopurine (BAP), 1,3-diphenylurea (DPU) and thidiazuron (TDZ) at five concentrations each (0.44, 1.33, 2.22, 3.11 and 4.44 µM/L) were tested for their effect on growth and development of *Origanum compactum* explants. Cytokinins free medium was considered a control.

2.4. Effect of Cytokinins Combined with Auxins

Three auxins: IAA (indole-3-acetic acid), NAA (1-naphthalene acetic acid) and IBA (indole-3-butyric acid) at four increasing concentrations (1.14, 2.85, 4.56 and 6.27 µM/L) were tested with the most appropriate cytokinin determined in the preceding test for the purpose of determining the best concentration of auxin that stimulate stem and root growth. The medium contains only cytokinin served as a double control.

2.5. Effect of Cytokinins and Auxins Combined with Gibberellic Acid

Five concentrations of gibberellic acid (0.29, 1.50, 2.60 and 2.89 µM/L) were tested with the best combination of cytokinin and auxin. The medium contain-

ing only cytokinin was considered the control medium number 1 and the medium supplemented with the best combination of cytokinin and auxin served as double control.

2.6. Effect of Cytokinins and Auxins Combined with Polyamines

Three polyamines (putrescine, spermidine and spermine) at five concentrations each (1.134, 3.402, 5.670, 7.938 and 11.340 µM), were tested with the best combination between cytokinin and auxin. The medium contains only cytokinin served as the control medium number 1 and the medium supplemented with the best combination of cytokinin and auxin serves as double control.

2.7. Culture Conditions

The tubes were hermetically wrapped with aluminum foil and autoclaved at 121˚C and a pressure of 1 bar for 21 mn. The cultures were incubated under specific conditions (photoperiod: 18/6h with 4000 lux light density, temperature: 24˚C ± 1˚C).

2.8. Acclimatization of Plantlets

The rooted explants, one-month old and about 15 cm were removed from the tubes and their roots were freed of the agar. They were transferred to plastic pots filled with autoclaved peat. The plantlets were covered with transparent plastic to prevent the loss of moisture and placed in a culture room (photoperiod: 18/6 h, humidity: 90% - 100%, temperature: 24˚C ± 1˚C). The leaves were sprayed with water twice a week. After four weeks, the transparent plastics were removed, and after a period of three weeks, the surviving ones were transferred to large pots and placed under the natural conditions of illumination and temperature. After ten days, the number of acclimated plants and the percentage of survival were determined.

2.9. Evaluation of Explant Growth

After 30-day culture, the following morphological measurements were evaluated:

—Mean explants length (cm),
—Mean number of buds,
—Mean number of shoots,
—Mean number of roots,
—Regeneration rate (%),
—Rooting rate (%),
—Hyperhydricity rate (%).

2.10. Statistical Analysis

36 explants were used for each experiment and data were processed by analysis of variance (ANOVA) to detect significant differences between means using the IBM SPSS 20 and Statistica 18 PSW software. Significant differences were

compared using Tukey's HSD. Values above $p \leq 0.05$ were considered significant.

3. Results

3.1. Effect of Macronutrients on Plantlets Growth

SD and $N_{30}K$ media favor bud multiplication (23.52 and 23.00), followed by MSm (18.74). On the other hand, minimal proliferation is noted for the SH and MS media (15.06 and 15.00). The maximum number of shoots is offered by Msm and B5 (1.66 and 1.60) followed by $N_{30}K$ (1.47) and the minimum number for SH (1.17). In addition, root multiplication is at its maximum for SD (9.14) followed by MSm (7.77) and SH (5.89) and at its minimum for $N_{30}K$ (3.77) (Table 1, Figure 1 and Figure 2). Furthermore, SD accords promising results in terms of elongation of the stem part (2.16 cm) followed by MS (1.86) and MSm (1.67 cm). On the other hand, the shortest shoots are generated by B5 (1.17 cm). (Table 1, Figure 1).

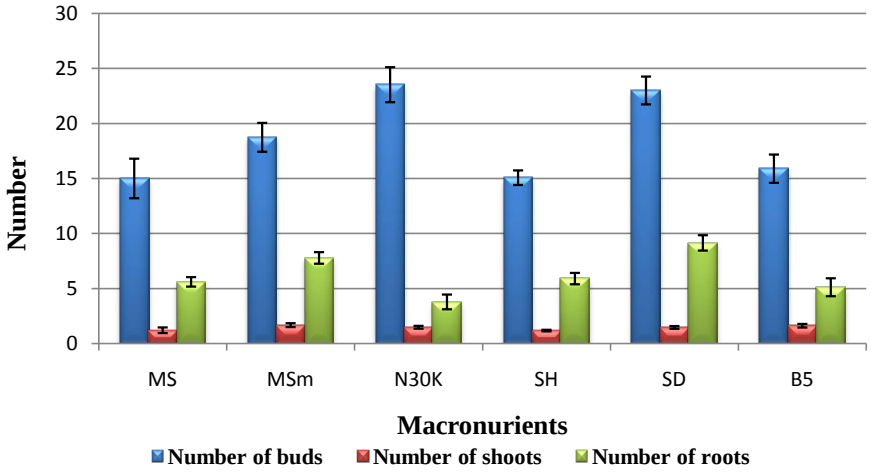


Figure 1. Effect of six macronutrients on the multiplication of buds, shoots and roots of *Origanum vulgare* L.

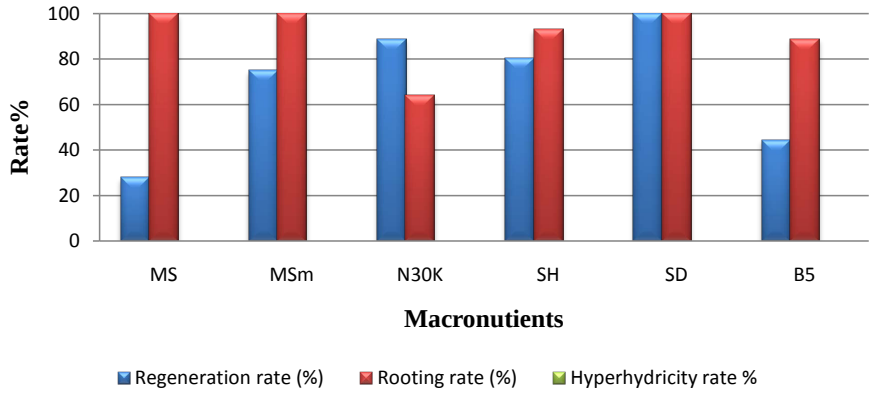


Figure 2. Effect of six macronutrients on the regeneration, rooting and hyperhydricity rates of *Origanum vulgare* L.

Table 1. Effect of six macronutrients on the micropropagation of *Origanum vulgare* L.

Medium	Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
MS	27.80	1.86 ± 0.14ab	15.00 ± 1.58b	1.20 ± 0.13ab	100.00	5.60 ± 0.73cd
MS_m	75.00	1.67 ± 0.09ab	18.74 ± 1.31ab	1.66 ± 0.17a	100.00	7.77 ± 0.52ab
$N_{30}K$	88.90	1.59 ± 0.11ab	23.52 ± 1.59a	1.47 ± 0.12ab	63.89	3.77 ± 0.67d
SH	80.55	1.43 ± 0.06b	15.06 ± 0.66b	1.17 ± 0.07b	93.10	5.89 ± 0.51bc
SD	100.00	2.16 ± 0.12a	23.00 ± 1.26a	1.45 ± 0.12ab	100.00	9.14 ± 0.69a
B_5	44.44	1.13 ± 0.08c	15.88 ± 1.28b	1.61 ± 0.16a	88.89	5.11 ± 0.81cd

Letters represent homogeneous groups; in each column, different letters indicate a significant difference at $p < 0.05$ using ANOVA and Tukey Post Hoc.

Thus, SD allows a total regeneration of the explants, followed by $N_{30}K$ (88.90%) and SH (80.55%). In addition, the vitro plants grown in MS all generate roots, as did MS and MSm. The absence of hyperhydric explants is noted in the six culture media (Table 1, Figure 3).

(a) (b)

Figure 3. Effect on macronutrients on the micropropagation of *Origanum vulgare* L. ((a) SD; (b) $N_{30}K$).

In conclusion, SD medium gives the best development and growth of vitroplants. It allows total regeneration and rooting of the explants and he was selected for the following experiments.

3.2. Effect of Cytokinins

Integration of cytokinins into the culture medium reveals notable changes, particularly in terms of bud, shoot and root multiplication (Table 2, Figure 4 and Figure 5).

Thus, the maximum number of buds is indicated in the case of the culture medium supplemented with 3.11 µM Kin (23.56) followed by the control medium (23.26) and that containing 2.22 µM Zeat (22.77), in contrast to the case of 4.44 µM TDZ and 1.33 µM adenine where a minimum multiplication is observed (13.63 and 13.86). In addition, the proliferation of shoots is at its peak for 4.44 µM adenine (1.907) followed by 2.22 and 1.33 µM BAP (1.84 and 1.81) while it is

at its lowest for 2.22 µM TDZ (1.30). Furthermore, the addition of different concentrations of Zeat has a positive effect on root multiplication. It is maximum for 2.22 µM Zeat (8.83) followed by 4.44 (8.00) and 3.11 µM Zeat (7.74). In opposition, TDZ at different concentrations is unfavorable to root proliferation. In addition, the maximum shoot elongation is noted for 3.11 µM Kin (2.87 cm), followed by 4.44 µM and 0.44 µM DPU (2.83 and 2.75 cm), and the minimum value is noted for 1.33 µM Zeat (1.20 cm) (Table 2, Figure 4).

Total rooting of explants is recorded for Kin at all concentrations, for Zeat at four concentrations (0.44, 1.33, 2.22 and 3.11 µM), 2ip at four concentrations (1.33, 2.22, 3.11 and 4.44 µM) and BAP at three concentrations (1.33, 2.22 and 4.44 µM). The highest rate of regeneration is reported for 4.44 µM DPU and 3.11 µM Kin (97.22% and 97.00%). However, low regeneration is noted with 4.44 µM BAP (22.22%) and hyperhydricity is observed at 0.44, 2.2 and 1.33 µM (3.00%, 6.25% and 3%, respectively) (Table 2, Figure 4).

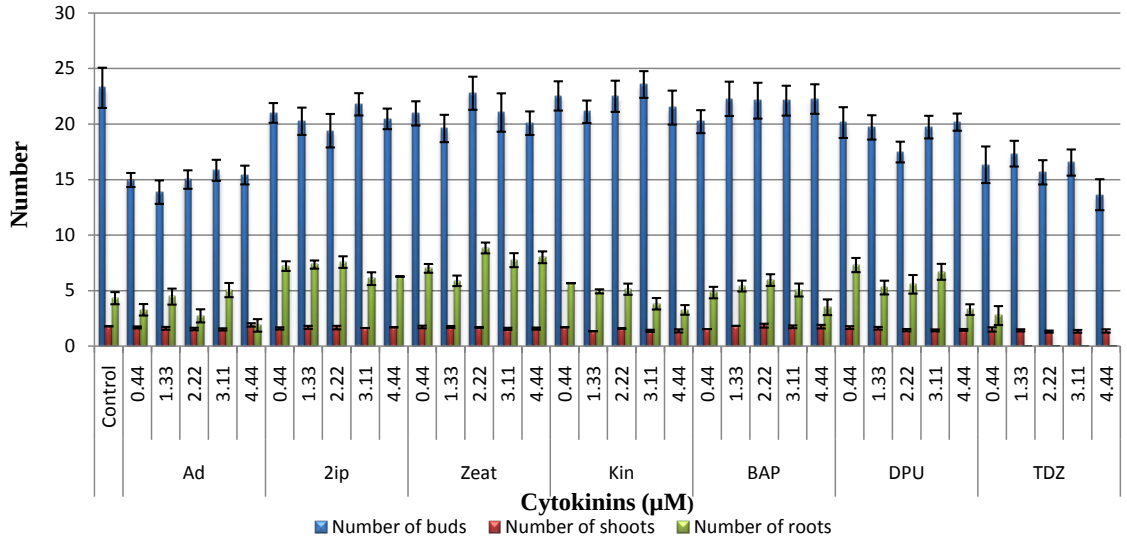


Figure 4. Effect of seven cytokinins at different concentrations on the multiplication of buds, shoots and roots of *Origanum vulgare* L.

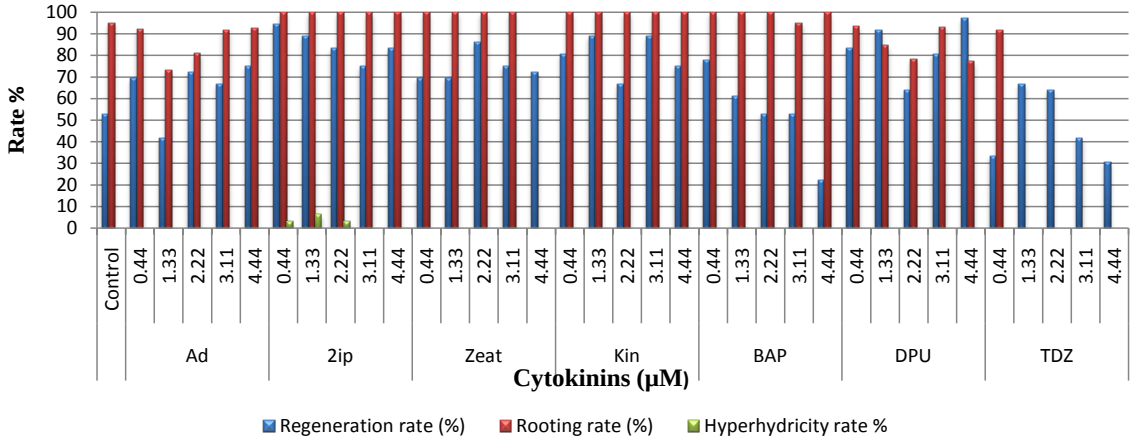


Figure 5. Effect of six macronutrients on the regeneration, rooting and hyperhydricity rates of *Origanum vulgare* L.

Table 2. Effect of cytokinins on the micropropagation of *Origanum vulgare* L.

Cytokinins (µM/L)		Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
Control (SD)		52.70	1.93 ± 0.17abcdef	23.26 ± 1.81a	1.78 ± 0.19a	95.00	4.31 ± 0.54efg
Ad	0.44	69.44	1.93 ± 0.11abcd	14.96 ± 0.63ab	1.68 ± 0.09 a	92.00	4.24 ± 0.51efg
	1.33	41.66	1.84 ± 0.12abcd	13.86 ± 1.05b	1.60 ± 0.13 a	73.33	3.86 ± 0.72fg
	2.22	72.22	1.92 ± 0.14abcd	15.00 ± 0.83ab	1.53 ± 0.11 a	80.76	4.23 ± 0.59efg
	3.11	66.66	2.32 ± 0.18ab	15.83 ± 0.94ab	1.50 ± 0.10 a	91.66	4.95 ± 0.64defg
	4.44	75.00	1.90 ± 0.15abcd	15.40 ± 0.84ab	1.90 ± 0.15a	92.59	4.88 ± 0.56defg
2ip	0.44	94.44	1.63 ± 0.09bcdef	21.00 ± 0.89a	1.58 ± 0.10a	97.00	7.20 ± 0.43abcd
	1.33	89.00	1.59 ± 0.10cdef	20.25 ± 1.23a	1.68 ± 0.13a	100.00	7.34 ± 0.36abc
	2.22	83.33	1.38 ± 0.07ef	19.40 ± 1.51ab	1.66 ± 0.16a	100.00	7.56 ± 0.52abc
	3.11	75.00	1.29 ± 0.07ef	21.77 ± 1.00a	1.62 ± 0 .09a	100,00	6.07 ± 0.57bcdef
	4.44	83.33	1.48 ± 0.12def	20.46 ± 0.92a	1.70 ± 0 .09a	100.00	6.26 ± 0.43bcde
Zeat	0.44	69.44	1.60 ± 0 .11bcdef	20.96 ± 1.09a	1.72 ± 0.12a	100.00	7.00 ± 0.39abcde
	1.33	69.44	1.20 ± 0.12f	19.60 ± 1.23ab	1.72 ± 0.09a	100.00	5.88 ± 0.47bcdef
	2.22	86.11	1.55 ± 0.07cdef	22.77 ± 1.49a	1.67 ± 0 .14a	100.00	8.83 ± 0.49a
	3.11	75.00	1.33 ± 0.10ef	21.03 ± 1.72a	1.55 ± 0.09a	100.00	7.74 ± 0.63ab
	4.44	72.22	1.38 ± 0.07def	20.07 ± 1.06a	1.57 ± 0.09a	96.10	8.00 ± 0.53ab
Kin	0.44	80.55	2.10 ± 0.14abc	22.53 ± 1.31a	1.70 ± 0.13a	100.00	5.66 ± 0.57bcdefg
	1.33	89.00	2.12 ± 0.15ab	21.10 ± 1.01a	1.34 ± 0.10a	100.00	4.93 ± 0.18defg
	2.22	66.70	1.98 ± 0.14abcd	22.50 ± 1.40a	1.58 ± 0.13a	100.00	5.12 ± 0.50defg
	3.11	97.00	2.87 ± 0.13a	23.56 ± 1.20a	1.57 ± 0.09a	100.00	3.81 ± 0.51fg
	4.44	75.00	2.48 ± 0.16ab	21.48 ± 1.53a	1.37 ± 0.14a	100.00	3.25 ± 0.43fg
BAP	0.44	77.80	1.36 ± 0 .08ef	20.21 ± 1.03a	1.53 ± 0.10a	90.00	4.82 ± 0.51defg
	1.33	61.11	1.28 ± 0.04ef	22.27 ± 1.54a	1.81 ± 0 .12a	100.00	5.40 ± 0.49bcdefg
	2.22	52.80	1.27 ± 0.07ef	22.10 ± 1.61a	1.84 ± 0.17a	100.00	5.94 ± 0.52bcdefg
	3.11	52.80	1.73 ± 0 .12def	22.10 ± 1.34a	1.73 ± 0.12a	95.00	5.05 ± 0.58cdefg
	4.44	22.22	1.08 ± 0.12ef	22.25 ± 1.33a	1.75 ± 0.16a	100.00	3.50 ± 0.70efg
DPU	0.44	83.33	2.75 ± 0.15a	20.13 ± 1.38a	1.66 ± 0.12a	93.33	7.30 ± 0.63abc
	1.33	91.66	2.40 ± 0.16ab	19.69 ± 1.09ab	1.60 ± 0.12a	84.84	5.27 ± 0.62bcdefg
	2.22	63.88	2.17 ± 0.20abc	17.47 ± 0.93ab	1.43 ± 0.10a	78.26	5.56 ± 0.83bcdefg
	3.11	80.55	2.41 ± 0.16ab	19.72 ± 1.01ab	1.41 ± 0.09a	93.10	6.68 ± 0.71bcde
	4.44	97.22	2.83 ± 0.21a	20.17 ± 0.77a	1.45 ± 0.08a	77.14	3.28 ± 0.48fg
TDZ	0.44	33.33	1.63 ± 0.12bcdef	16.33 ± 1.64ab	1.50 ± 0.19a	91.66	2.75 ± 0.85fg
	1.33	66.66	1.80 ± 0.06abcd	17.33 ± 1.15ab	1.41 ± 0.10a	8.33	0.00 ± 0.00g
	2.22	63.88	1.75 ± 0.07def	15.65 ± 1.09ab	1.30 ± 0.09a	0.00	0.00 ± 0.00g
	3.11	41.66	1.78 ± 0.08def	16.53 ± 1.17ab	1.33 ± 0.12a	0.00	0.00 ± 0.00g
	4.44	30.55	1.81 ± 0.18abcd	13.63 ± 1.39b	1.36 ± 0.15a	0.00	0.00 ± 0.00g

Letters represent homogeneous groups; in each column, different letters indicate a significant difference at $p < 0.05$ using ANOVA and Tukey Post Hoc.

In conclusion, the medium supplemented with 3.11 µM Kin is the most favorable for the micropropagation of vitroplants of *Origanum vulgare*, since it ensures high regeneration, optimal elongation and total rooting of the explants as well as maximum multiplication of the buds with the absence of hyperhydric explants. The root part will be improved by combining 3.11 µM Kin and three auxins at different concentrations (Figure 6).

(a) (b) (c) (d)

Figure 6. Effect of cytokinins on micropropagation of *Origanum vulgare* L. ((a) Control; (b) 3.11 µM Kin; (c) 1.33 µM DPU; (d) 3.11 µM DPU).

3.3. Effect of Auxins

The combination of 3.1 µM Kin and the three auxins results in changes in the *in vitro* growth of *Origanum vulgare* explants, particularly at the root part (Table 3, Figure 7 and Figure 8).

Table 3. Effect of auxins combined with 3.11 µM Kin on the micropropagation of *Origanum vulgare* L.

Auxins (µM/L)		Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
Control 1 SD		52.70	1.93 ± 0.17d	23.26 ± 1.81abc	1.78 ± 0.19ab	95.00	4.31 ± 0.54bcd
Control 2 (3.11 µM Kin)		89.00	2.07 ± 0.13cd	23.56 ± 1.20abc	1.37 ± 0.09b	100.00	3.81 ± 0.51cd
IAA	1.14	83.33	2.78 ± 0.16bcd	24.60 ± 1.22ab	1.63 ± 0.12ab	100.00	6.13 ± 0.41abc
	2.85	83.33	2.94 ± 0.19bcd	23.20 ± 1.20abc	1.46 ± 0.09ab	100.00	6.43 ± 0.64abc
	4.56	94.44	2.22 ± 0.16cd	25.55 ± 1.48ab	1.91 ± 0.14a	91.17	6.41 ± 0.67abc
	6.27	100.00	2.43 ± 0.17bcd	25.72 ± 1.25ab	1.63 ± 0.11ab	100.00	7.16 ± 0.58ab
IBA	1.14	77.80	2.81 ± 0.25bcd	21.10 ± 1.20bc	1.46 ± 0.09ab	96.42	5.82 ± 0.62abc
	2.85	93.10	2.91 ± 0.22bcd	23.10 ± 1.68abc	1.58 ± 0.09ab	80.50	5.31 ± 0.60abcd
	4.56	63.88	3.07 ± 0.18bc	17.04 ± 0.931c	1.34 ± 0.10ab	100.00	6.21 ± 0.69abc
	6.27	100.00	4.20 ± 0.27a	28.11 ± 1.53a	1.77 ± 0.09ab	94.44	7.72 ± 0.66a
NAA	1.14	94.44	2.66 ± 0.19bcd	23.29 ± 0.19.abc	1.55 ± 0.10ab	91.17	4.50 ± 0.51c
	2.85	94.44	2.56 ± 0.16bcd	23.21 ± 1.63abc	1.57 ± 0.14ab	79.41	2.84 ± 0.41d
	4.56	66.66	2.43 ± 0.15bcd	22.64 ± 1.20abc	1.60 ± 0.10ab	69.44	4.12 ± 0.38cd
	6.27	85.18	3.17 ± 0.25d	23.70 ± 1.80abc	1.70 ± 0.12ab	75.00	4.62 ± 0.65bcd

Letters represent homogeneous groups; in each column, different letters indicate significant difference at $p < 0.05$ using ANOVA and Tukey Post Hoc.

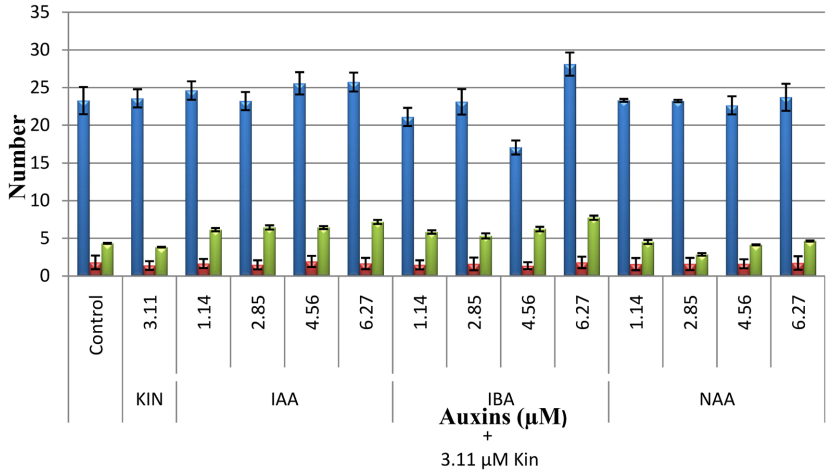


Figure 7. Effect of auxins combined with 3.11 µM Kin at different concentrations on the multiplication of buds, shoots and roots of *Origanum vulgare* L.

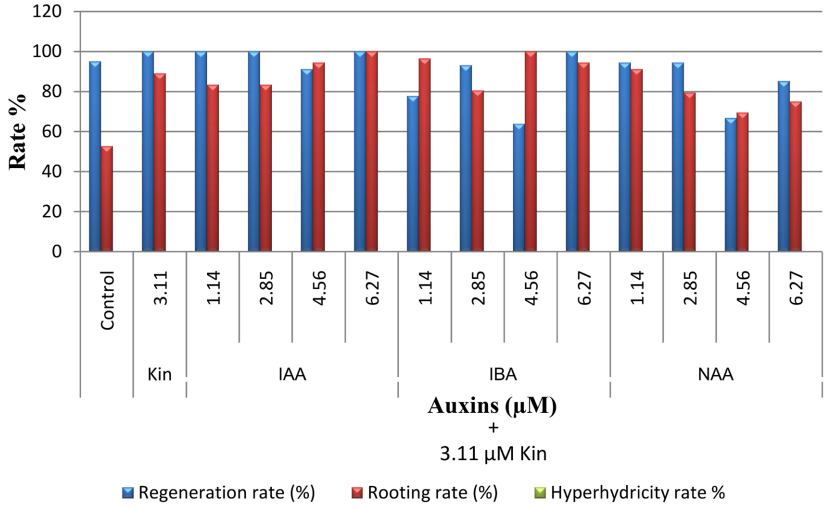
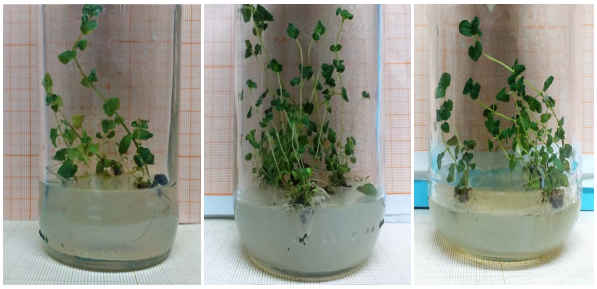


Figure 8. Effect of auxins combined with 3.11 µM Kin at different concentrations on the regeneration, rooting and hyperhydricity rates of *Origanum vulgare* L.

Thus, the maximum number of roots is noted in the case of the medium supplemented with 6.27 µM AIB (7.72), followed by 6.27 and 2.85 µM IAA (7.16 and 6.41), unlike the medium free of growth regulators where multiplication is minimal (0.55). Similarly, bud proliferation is at its maximum for 6.27 µM IBA (28.11), followed by 6.27 µM IAA (25.72) and 4.56 µM IAA (25.55), and is at its minimum for 4.56 µM IBA (17.04). Therefore, there is no significant difference for shoot multiplication, the maximum value being noted for 4.56 µM IAA (1.91) and the minimum shoot multiplication recorded for 4.56 µM IBA (1.34) (**Table 3**, **Figure 7**).

Compared with the control medium without growth regulators, the combination of 3.11 µM Kin with 6.27 µM AIB is suitable for elongation of the aerial part of the explants (4.20 cm), followed by 6.27 µM NAA and 4.56 µM IBA (3.17 and 3.07 cm) (**Table 3**, **Figure 8**).

Total rooting of explants is indicated for 3.11 µM Kin, 3.11 µM Kin + 1.14 µM IAA, 3.11 µM Kin + 2.85 µM IAA, 3.11 µM Kin + 6.27 µM IAA and 3.11 µM Kin + 4.56 IBA µM. In addition, the medium supplemented with 3.11 µM Kin + 6.27 µM IBA allows total regeneration of the vitroplants. No cases of hyperhydricity are observed in the different combinations (Figure 9).



(a) (b) (c)

Figure 9. Effect of auxins combined with 3.11 µM Kin on micropropagation of *Origanum vulgare* L. ((a) 4.56 µM IBA, (b) 4.56 µM IAA, (c) 6.27 µM NAA).

3.4. Effect of Cytokinins and Auxins Combined with Gibberellic Acid

The combination of different concentrations of AG_3 with 3.11 µM Kin and 6.27 µM IBA leads to some changes in the *in vitro* growth of *Origanum vulgare* explants (Table 4, Figure 10 and Figure 11).

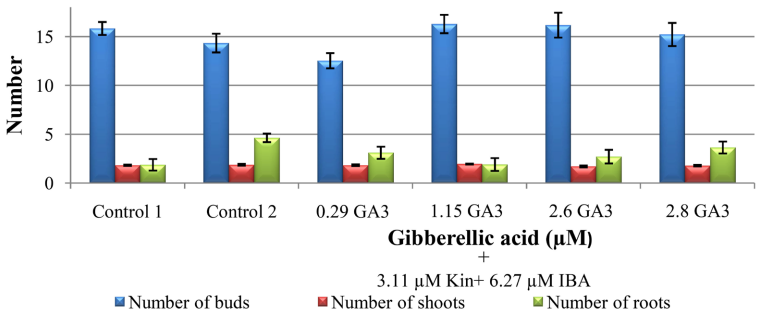


Figure 10. Effect of GA_3 at four concentrations combined with 3.11 µM Kin and 6.27 µM IBA on the multiplication of buds, nodes and roots of *Origanum vulgare* L.

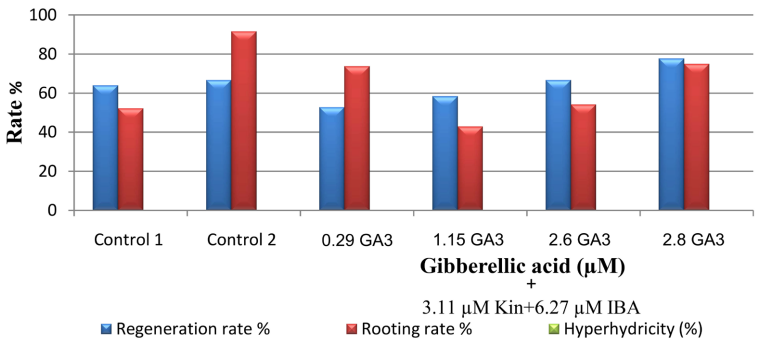


Figure 11. Effect of GA_3 at four concentrations combined with 3.11 µM Kin and 6.27 µM IBA on the regeneration, rooting and hyperhydricity rates of *Origanum vulgare* L.

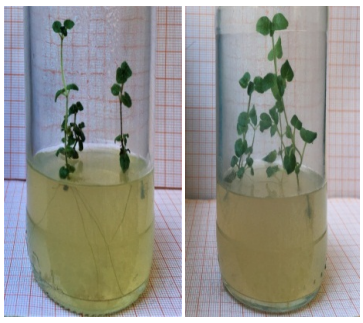
Table 4. Effect of gibberellic acid combined with 3.11 µM Kin and 6.27 µM IBA on the micropropagation of *Origanum vulgare* L.

		Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
Control 1 (SD)		66.66	1.16 ± 0.28ab	15.82 ± 0.95a	1.82 ± 0.08a	91.66	1.86 ± 0.43b
Control 2 (3.11 µM Kin + 6.27 µM IBA)		63.88	1.59 ± 0.16a	14.33 ± 0.66a	1.87 ± 0.06a	52.17	4.62 ± 0.59a
GA_3 (µM/L)	0.29	52.77	1.37 ± 0.22ab	12.52 ± 0.77a	1.82 ± 0.08a	73.68	3.10 ± 0.62ab
	1.5	58.33	0.45 ± 0.13b	16.28 ± 0.93a	1.95 ± 0.04a	42.85	1.90 ± 0.65b
	2.60	66.66	0.89 ± 0.24ab	16.11 ± 1.27a	1.70 ± 0.09a	54.16	2.70 ± 0.70ab
	2.89	77.78	1.56 ± 0.23a	15.21 ± 1.18a	1.78 ± 0.07a	75.00	3.64 ± 0.60ab

Letters represent homogeneous groups; in each column, different letters indicate significant difference at $p < 0.05$ using ANOVA and Tukey Post Hoc.

Thus, the addition of GA_3 acts positively on the multiplication of buds. The maximum number is noted in the case of 1.5 µM GA_3 (16.28) followed by 2.60 µM GA_3 (16.16) and followed by the control medium 1 (15.82). In contrast, a low concentration of GA_3 (0.29 µM) allows minimal bud proliferation (12.52). With regard to shoot multiplication, no significant differences are observed between the different combinations and the two-control media; an increase in number is noted in the case of 1.5 µM GA_3 (1.95) followed by the control medium 2 (3.11 µM Kin + 6.27 µM IBA) and control medium 1 (SD only) (1.87 and 1.82 respectively) while a decrease is noted in the case of 2.60 µM GA_3 (1.70). Moreover, root multiplication is at its maximum in the case of control medium 2 (4.62) followed by 2.89 µM GA_3 (3.64) and 0.29 µM GA_3 (3.10) while it is at its minimum in the case of 1.5 µM GA_3 (1.90). On the other hand, an optimum concentration of GA_3 (2.89 µM GA_3) is favorable for the elongation of the stem part (2.41 cm); however, the maximum value is noted for control medium 2 (3.11 µM Kin + 6.27 µM IBA) (2.76 cm), and the minimum for GA_3 (0.29 µM GA_3) (1.63 cm) (Table 3, Figure 10).

In addition, the control medium 2 (3.11 µM Kin + 6.27 µM IBA) generates a high level of rooted vitroplants (91.66%) followed by the medium supplemented with 2.89 and 0.29 µM GA_3 (75.00 and 73.68%, respectively). On the other hand, the medium supplemented with 2.89 µM GA_3 allows a high percentage of regeneration (77.78%) followed by the control medium 2 and that supplemented with 2.60 µM GA_3 (66.66%) (Table 3, Figure 11 and Figure 12).



(a) (b)

Figure 12. Effect of GA_3 combined with 3.11 µM Kin and 6.27 µM IBA on micropropagation of *Origanum vulgare* L. ((a) 0.29µM GA_3, (b) 1.15 µM GA_3).

3.5. Effect of Polyamines Combined with Cytokinins and Auxins

The combination of three polyamines at different concentrations with 3.11 µM Kin and 6.27 µM IBA results from some changes in the micropropagation of *Origanum vulgare* vitroplants (Table 5, Figure 13 and Figure 14).

Thus, an increase in the number of buds is noted in the case of 1.134 µM Putrescine (21.72) followed by the control medium 2 (3.11 µM Kin + 6.27 µM IBA) (21.44) and 5.67 µM spermine (21.38). On the other hand, a decrease in number is reported in the case of 7.938 µM spermidine (14.08). For what concerns the shoots multiplication, it is at its maximum in the case of 5.67 µM and 1.134 µM putrescine (2.07 and 2.03) followed by 11.34 µM putrescine (1.94) while it is at its minimum in the case of 7.938 µM spermidine (1.44). In addition, in comparison with the control medium 2 (3.11 µM Kin + 6.27 µM AIB), the addition of the polyamines, with the exception of 1.134 µM Putrescine where a maximum proliferation of the roots is marked (5.22), has an insignificant effect on the root multiplication; in fact, it is minimal for 5.67 µM spermidine (0.33) and absent for 3.402 µM putrescine and 3.402 µM spermidine. On the other hand, the longest explants are regenerated in the medium supplemented with 1.134 µM spermidine (4.21 cm) followed by those cultivated in the medium supplemented with 7.938 µM spermine and 3.402 µM spermidine (3.50 cm) whereas the shortest explants are indicated in the case of 5.67 µM Spermidine (2.38 cm) (Table 5, Figure 13).

Table 5. Effect of polyamines combined with 3.11 µM Kin and 6.27 µM IBA on the micropropagation of *Origanum vulgare* L.

Polyamines (µM/L)		Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
Control 1 (3.11 µM Kin)		73.33	3.16 ± 0.29ab	17.73 ± 0.99ab	1.80 ± 0.10ab	41.60	2.73 ± 0.65abcde
Control 2 (3.11 µM Kin + 6.27 µM IBA)		94.44	3.46 ± 0.28ab	21.44 ± 2.20a	1.83 ± 0.20ab	50.00	4.27 ± 0.69ab
Putrescine	1.134	95.45	3.32 ± 0.33ab	21.72 ± 1.75a	2.04 ± 0.13a	61.11	5.22 ± 0.53a
	3.402	0.00	3.13 ± 0.38ab	17.81 ± 1.71ab	1.54 ± 0.15ab	30.56	0.00 ± 0.00e
	5.67	78.94	2.74 ± 0.19b	19.57 ± 1.55ab	1.68 ± 0.13ab	52.78	3.21 ± 0.59ab
	7.938	78.51	3.16 ± 0.33ab	18.71 ± 2.34	1.57 ± 0.13ab	38.89	3.35 ± 0.70abc
	11.34	27.78	2.68 ± 0.19ab	20.56 ± 1.75ab	1.94 ± 0.17ab	50.00	1.00 ± 0.45de
Spermidine	1.134	52.94	4.21 ± 0.36a	18.11 ± 0.89ab	1.52 ± 0.12ab	47.22	1.64 ± 0.41bcde
	3.402	0.00	3.50 ± 0.27ab	15.71 ± 1.06ab	1.47 ± 0.11ab	58.46	0.00 ± 0.00e
	5.67	27.78	2.38 ± 0.21b	16.00 ± 1.04ab	1.56 ± 0.12ab	50.00	0.33 ± 0.14cde
	7.938	28.00	3.38 ± 0.26ab	14.80 ± 0.82b	1.44 ± 0.10b	69.44	1.04 ± 0.43cde
	11.34	33.33	2.97 ± 0.25ab	21.06 ± 1.87ab	1.80 ± 0.14ab	41.67	1.06 ± 0.45bcde
Spermine	1.134	68.18	3.16 ± 0.20ab	19.09 ± 1.09ab	1.59 ± 0.10ab	61.11	2.68 ± 0.67abcd
	3.402	60.00	3.34 ± 0.27ab	16.70 ± 1.06ab	1.55 ± 0.11ab	55.56	1.50 ± 0.36bcde
	5.67	53.84	2.56 ± 0.24b	21.38 ± 1.85ab	2.07 ± 0.17a	36.11	0.92 ± 0.28bcde
	7.938	57.14	3.68 ± 0.40ab	20.57 ± 1.84ab	1.57 ± 0.13ab	38.89	2.35 ± 0.82abcd
	11.34	40.90	3.41 ± 0.26ab	16.45 ± 1.06ab	1.50 ± 0.10ab	61.11	1.45 ± 0.47cde

Letters represent homogeneous groups; in each column, different letters indicate significant difference at $p < 0.05$ using ANOVA and Tukey Post Hoc.

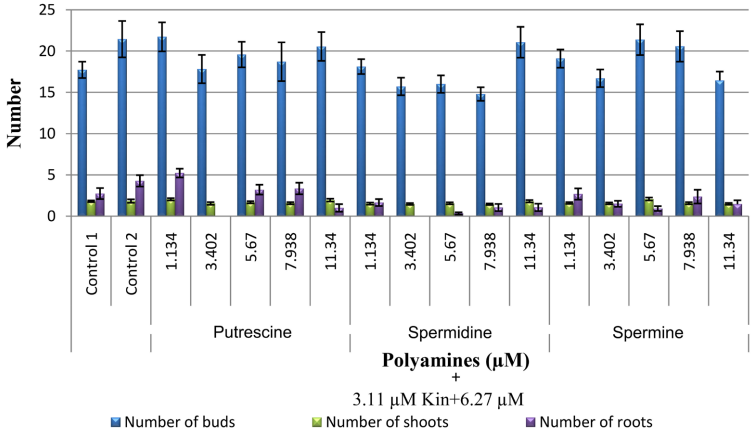


Figure 13. Effect of 3 polyamines at 5 concentrations combined with 3.11 µM Kin and 6.27 µM IBA on the multiplication of buds, nodes and roots of *Origanum vulgare* L.

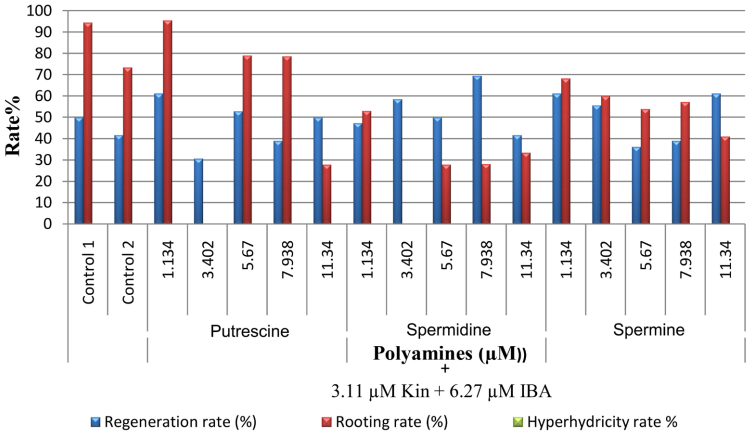


Figure 14. Effect of 3 polyamines at 5 concentrations combined with 3.11 µM Kin and 6.27 µM IBA on the regeneration, rooting and hyperhydricity rates of *Origanum vulgare* L.

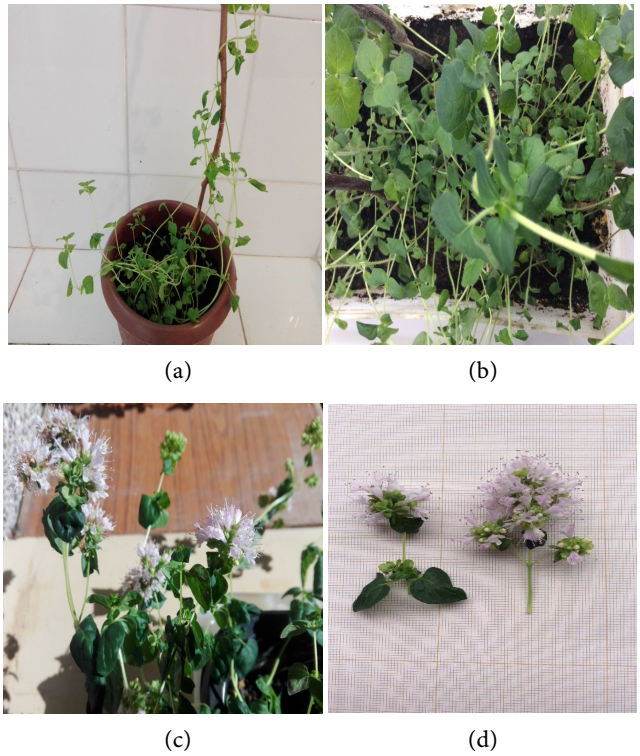
The percentage of regeneration is relatively high in the case of 1.134 µM putrescine, 7.938 µM spermidine, 1.134 and 11.34 µM spermine (61.11%). In addition, the rhizogenesis rate is maximal in the case of control medium 2 and 1.134 µM putrescine (94.44%). No cases of hyperhydria are noted in the various combinations (Table 5, Figure 14 and Figure 15).

(a) (b)

Figure 15. Effect of polyamines combined with 3.11 µM Kin and 6.27 µM IBA on micropropagation of *Origanum vulgare* L. ((a) 7.938 µM Spd; (b) 5.67 µM Sp).

3.6. Acclimatization of Plantlets

Explants grown in SD medium supplemented with 3.11 µM Kin and 6.27 µM IBA (the best medium for rooting) showed good root and foliar development, and the survival percentage of seedlings acclimatized in the culture room, and after their transfer under natural conditions, was 96% (**Figure 16**).



(a) (b)

(c) (d)

Figure 16. Acclimatization phase of *Origanum vulgare* L. ((a) Acclimatization after 3 months; (b) after 5 months; (c) after 7 months, (d) inflorescences).

4. Discussion

The evaluation of the effect of macronutrients on the micropropagation of *Origanum vulgare* allowed us to choose the SD culture medium since it ensured total regeneration of vitroplants, a relatively high percentage of rooted plants and a better multiplication of buds and roots. It also offered promising results in terms of root multiplication. However, in numerous investigations of the micropropagation of *Origanum vulgare*, MS medium was the most preferred [23]-[28]. In addition, Morone-Fortunato and Avato (2008) [29] chose a less concentrated medium (Nitsh medium) in their protocol for the *in vitro* culture of *Origanum vulgare*. On the other hand, Economou *et al.* (2011) [30] established explants culture on B5 medium.

Improvements in vitroplants growth are observed following the addition of cytokinins to specific concentrations in the culture medium. Thus, an optimization in terms of elongation of the vegetative part is noted with 3.11 µM Kin, 4.44 and 0.44 µM DPU. The addition of 1.33, 3.11 and 0.44 µM DPU allowed better

development of the root part. In addition, shoot multiplication improved with the integration to the culture medium 4.44 µM adenine and 2.22 µM BAP. In addition, high concentrations of zeatin are favorable for root propagation. Optimal regeneration of the vitroplants is noted with 3.11 µM Kin and 4.44 µM DPU. Total rooting is observed with different concentrations of Kin, Zeat (0.44, 1.33, 2.22, and 3.11 µM), 2ip (1.33, 2.22, 3.11, and 4.44 µM) and BAP (1.33, 2.22, and 4.44 µM). Hyperhydricity is marked only as a low percentage at 0.44, 2.22, and 1.33 µM (3, 6.25 and 3%, respectively).

Other studies have examined the effect of cytokinin concentration and type, including BAP on the growth and development of *Origanum vulgare* explants. Thus, Pandey *et al*. (2019) [27] demonstrated that the cytokinin-free control medium caused browning of explants and shoot proliferation was not triggered. However, a maximum elongation of the shoots is noted in the case of the medium supplemented with 4 µM BAP. On the other hand, the presence of high concentration of BAP in the culture medium has a negative effect on the induction of shoots. Morone-Fortunato and Avato (2008) [29] reported that 5.94 µM BAP allowed multiple shoots regeneration of *Origanum vulgare* subsp. *hirtum* explants. In addition, Cristea *et al*. (2008) [24] found that BAP from 2.97 to 5.94 µM had the best results in terms of proliferation and regeneration of *Origanum vulgare* shoots. In opposition, its replacement with Kinetin did not promote explant regeneration, unlike our study. Therefore, the study of the effect of cytokinin concentration and type on organogenesis is reported in several species belonging to the genus *Origanum*. These results reveal the importance of these chemicals in the micropropagation and reproduction of plants, because they promote cell divison in plant roots and shoots and also axillary bud growth and affect apical dominance in appropriate concentartion. Korkor *et al*. (2017) [31] showed that 5.94 µM BAP had a negative effect on the elongation of *Origanum majorana* shoots and a positive effect on their multiplication. Abdallah *et al*. (2017) [32] demonstrated that treatments with 2 µM Kinetin allowed good results in terms of proliferation shoot elongation of *Origanum syriacum* L explants. Moreover, Zayova *et al*. (2019) [33] found that 2.28 and 4.56 µM Zeatin allow the formation of multiple shoots of Greek oregano (*Origanum heracleoticum* L.).

In our study, the medium supplemented with 3.11 µM Kin was most suitable for improving the growth of vitroplants of *Origanum vulgare*, particularly the vegetative part. The root part is optimized by combining 3.11 µM Kin with three auxins at precise concentrations. In fact, the integration of auxins ensured not only the improvement of the development of the roots, but also of the vegetative part, in particular with 6.27 µM IBA where an optimal multiplication of buds and roots is observed as well as a better elongation of shoots. On the other hand, the increase in NAA concentration is unfavorable for root development, in agreement with the study of Cristea *et al*. (2008) [24] where rooting medium containing low concentration of NAA showed good development of *Origanum*

vulgare explants. The cytokinin/auxin combination sometimes only leads to the development of the vegetative or root part. In the investigation of Pandey *et al.* (2019) [27], the presence of high-concentration BAP with 0.25 µM NAA ensured a better multiplication of shoots while rooting is initiated in the medium supplemented with only 50 µM IBA. Similarly, Cristea *et al.* (2008) [24] proved that the combination of 8.88 µM BAP and 0.54 µM NAA guarantees a high percentage of regeneration and maximum shoot proliferation, while a high rate of rooted explants occurs in the medium supplemented with 3.22 µM NAA without any combination with cytokinins. The integration of auxins without combination with cytokinins into the culture medium is sometimes sufficient for the development of both parts. In this context, Nanova and Slavova (2006) [34] showed that the medium supplemented with only 0.85 µM IAA gave better results in terms of vegetative part development with an optimal percentage of vitroplants of *Origanum vulgare* subsp. *hirtum* rooted. On the other hand, the species response varies according to the cytokinin/auxin ratio in the study of Camacho *et al.* (2018) [5] where 8.88 µM BAP/10.70µM NAA ratio regenerate vitroplants with better morphological characteristics. Nicuță and Lazar (2018) [26] showed that 4.44 µM BAP/6.27µM IAA ratio ensured rapid explant growth and good root development, while 4.44 µM BAP/2.68µM NAA ratio provided vitroplants with thin and short roots.

In our study, the ratio of 3.11 µM Kin/6.27µM IBA is the most suitable for the development and growth of both the root and aerial parts and the combination of three polyamines at different concentrations with this ratio alters the growth of *Origanum vulgare*. Compared with the two-control media, low-concentration of putrescine is considered to be the most suitable for *in vitro* culture of explants, as it allows optimal bud and root multiplication, as well as a high rate of regeneration and rhizogenesis and absence of hyperhydria. In addition, spermine at 5.67 µM allowed maximum shoot multiplication and 1.134 µM spermidine contributed to a better elongation of vitroplants. In contrast, the combination of 3.402 µM putrescine and spermidine with 3.11 µM Kin + 6.27 µM IBA had an inhibitory effect on root development. The study of the effect of polyamines alone or in combination with cytokinins or/and auxins has been addressed in investigations of other species belonging to the Lamiaceae family. El Ansari *et al.* (2020) [35] showed that medium supplemented with 10 µM Spermine gave the best results in terms of bud, shoot and root multiplication, and Sarropoulou and Maloupa (2019) [36] showed that the integration of exogenous polyamines contributed to numerous modifications in the growth of *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri*. Thus, among the three polyamines tested, spermidine gave the best results in terms of shoot and root multiplication, while spermine allowed maximum shoot elongation compared with spermidine and putrescine. In addition, 10 µM Spermidine is most suitable for shoot proliferation. However, 1 µM spermine is most favorable for the elongation of the vegetative part. For the root part, 50 µM Spermidine provided the best propagation of

roots. This is in contrast to the study of Erland and Mahmoud (2014) [37] which showed that treatments with spermidine and spermine had an inhibitory effect on rooting, a total absence of rooted vitroplants in the medium supplemented with 1 Mm spermine, and browning and necrosis in the presence of high polyamine concentrations on *Lavandula* x *intermedia* cv Grosso explants. Moreover, Carlos Sánchez-Gras and Segura (1988) [38] proved that the effect of Spermidine depends on its concentration in the culture medium and the type of explant: for cotyledons, 0.01 mM spermidine has a rhizogenesis-promoting effect, while high-concentration of spermidine inhibits the NAA response to root induction; for hypocotyls, the combination of 0.01 mM spermidine and 27 µM NAA increases root induction. Oliveira *et al.* (2019) [39] showed that among the polyamines tested on the *in vitro* growth of *Lavandula angustifolia* L. vitroplants, 5,67 µM of putrescine and spermidine promote the *in vitro* culture of explants. Frabetti *et al.* (2009) [40] incorporated exogenous polyamines into the culture medium and combined them with auxins to optimize the rooting of *Teucrium fruticans* L. and they observed that putrescine alone or in combination with IBA does not improve root multiplication and rhizogenesis rate. The combination of polyamines and cytokinins was tested by Fazal *et al.* (2016) [41]; these authors showed that the combination of Kinetin and 11.34 µM putrescine induced elongation of the vegetative part and maximum shoot proliferation.

GA_3 combined with 3.11 µM Kin + 6.27 µM IBA improves the growth of *Origanum vulgare* vitroplants, especially in the propagation phase for the vegetative part. Thus, the maximum number of buds and shoots is given with 1.15 µM GA_3. However, the presence of high concentrations of GA_3 in the culture medium has a negative effect on bud multiplication. On the other hand, it favors shoot elongation and root development and growth. An optimal concentration of GA_3 (2.89 µM) allows a relatively high rate of rhizogenesis and maximum regeneration of the vitroplants. Few studies on the effect of gibberellic acid combined with or without auxins are reported in *Origanum* genus. Harfi *et al.* (2019) [42] showed maximum elongation of *Origanum glandulosum* Desf. in a medium supplemented with 2.22 µM BAP and 1.44 µM GA_3, and the combination of 2.22 µM BAP, 1.42 µM IAA and 1.44 µM GA_3 induced callus formation; they also showed that the combination of GA_3 with IAA without cytokinin addition was favorable for root development and allowed the regeneration of a high percentage of rooted vitroplants. Goleniowski *et al.* (2003) [16] noted that the replacement of NAA with 0.25 µM GA_3 and its combination with BAP showed a positive response for shoot elongation of vitroplants of "Mendocino" oregano, but in return the multiplication decreased. Sevindik *et al.* (2017) [43] reported that the combination of 2.22 µM BAP and 0.58 µM GA_3 generates a maximum number of shoots and is the most efficient for the micropropagation of *Origanum sipyleum* L.

Integration of GA_3 alone or in combination with cytokinins or/and auxins is reported in several species of Lamiaceae. Arumugam *et al.* (2020) [44] found that

the combination of 4.44 µM BAP and 2.89 µM GA_3 allowed maximum elongation and shoot multiplication of *Plectranthus amboinicus* (Lour.) Sprengel, but generated a high percentage of vitroplants with abnormal morphological characters. In the micropropagation of five Lamiaceae, *Thymus syriacus* Boiss., *Clinopodium insulare* (Candargy) Govaerts, *Clinopodium serpyllifolium* subsp. *fruticosum* (L.) Bräuchler, *Origanum majorana* L. and *Thymbra capitata* (L.) Cav., the medium supplemented with 4.65 µM Kin and 0.87 µM GA_3 is shown to be the best for shoot multiplication and proliferation for *Clinopodium insulare* (96%) [45]. Ozudogru *et al.* (2011) [46] demonstrated that the medium supplemented with 4.65 µM Kin and 0.87 µM GA_3 ensured optimum shoot proliferation and maximum regeneration rate of *Thymus longicaulis* C.Presl and *T. vulgaris* L. vitroplants. Also, Sevindik and Tutuncu (2020) [47] showed that the combinations 8.88 µM BAP + 5.77 µM GA_3 and 8.88 µM BAP + 4.32 µM GA_3 are favorable for shoot multiplication of *Lamium garganicum* L. subsp. *striatum* (Sm.) Hayek var. *striatum*.

5. Conclusions

This study represents a well-detailed and original protocol for the micropropagation of *Origanum vulgare* explants stored in the Tetouan Plant Biotechnology Laboratory.

Initially, the SD medium was chosen because it provided total regeneration of vitroplants, a relatively high percentage of rooted plants, and improved bud and root propagation. The SD medium also offered promising results in terms of vegetative elongation. After the multiplication of cultures, the evaluation of the effect of seven cytokinins at five concentrations showed that Kinetin at 3.11 µM allows high regeneration of vitroplants, optimal elongation, maximum bud multiplication, and total rooting of explants and absence of hyperhydric vitroplants. The addition of 6.27 µM IBA with 3.11 µM Kinetin allowed not only the improvement of the root part development, but also of the vegetative part with an optimal multiplication of buds and roots. The combination of polyamines with 3.11 µM Kinetin and 6.27 µM IBA shows that low-concentration of putrescine is most suitable for *in vitro* culture of explants. It allows optimum propagation of buds and roots, as well as a high rate of regeneration and rhizogenesis and absence of hyperhydria. The incorporation of GA_3 into the culture medium supplemented with 3.11 µM Kinetin and 6.27 µM IBA allowed the growth of *Origanum vulgare* vitroplants to be improved, especially in the propagation phase of the vegetative part. Thus, the maximum number of buds and shoots is high in the case of 1.15 µM GA_3. Finally, acclimatization was successfully established by the use of vitroplants that showed good foliar and root development. Two months after acclimatization, 100% of the plants were in good condition, and after 7 months, acclimatized plants developed flowers between May and July.

From a practical point of view, the protocol described makes it possible to overcome conclusively the decreasing of wild *Origanum vulgare* and regenerate

plants that can meet market needs for essential oils and bioactive compounds.

With regard to plant growth regulators, another chemicals could be used to prompt the growth of *Origanum vulgare* L., as like the elicitors, which notably include yeast extract, methyl jasmonate, salicylic acid, vanadyl sulphate and chitosan.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] eFlore (2021) Tela Botanica. https://www.tela-botanica.org/bdtfx-nn-46407-synthese

[2] Ietswaart, J.H. (1980) A Taxonomic Revision of the Genus *Origanum* (Labiatae). Springer, Dordrecht. https://doi.org/10.1007/978-94-009-9156-9 https://www.springer.com/gp/book/9789060214633

[3] Şahin, F., Güllüce, M., Daferera, D., Sökmen, A., Sökmen, M., Polissiou, M. and Agar, G. and Özer, H. (2004) Biological Activities of the Essential Oils and Methanol Extract of *Origanum vulgare* ssp. *vulgare* in the Eastern Anatolia Region of Turkey. *Food Control*, **15**, 549-557. https://doi.org/10.1016/j.foodcont.2003.08.009

[4] Alarcón, T. and Esther, M. (2014) Evaluación de la actividad antioxidante del aceite esencial foliar extraído de especies de orégano (*Origanum vulgare*), orégano "borde blanco" (*Origanum vulgare* ssp) y oreganito (*Lippia alba*) cultivado en La zona Norte del departamento de Bolívar (Colombia). Trabajo de grado—Maestría, Universidad Nacional de Colombia Sede Medellin Facultad de Ciencias Agropecuarias Departamento de Ingeniera Agricola y de Alimentos.

[5] Camacho, H.J.C., Zambrano, C.A.C. and Rodríguez, L.G. (2018) Evaluation of the *in Vitro* Growth of the Culture of Oregano (*Origanum vulgare*) from Organogenesis's Technology. *Respuestas*, **23**, 36-42. https://doi.org/10.22463/0122820X.1726 https://revistas.ufps.edu.co/index.php/respuestas/article/download/1726/1882

[6] Raina, A.P. and Negi, K.S. (2014) Chemical Diversity among Different Accessions of *Origanum vulgare* L. ssp. *vulgare* Collected from Central Himalayan Region of Uttarakhand, India. *Journal of Essential Oil Research*, **26**, 420-426. https://doi.org/10.1080/10412905.2014.948969

[7] Chishti, S., Kaloo, Z.A. and Sultan, P. (2013) Medicinal Importance of Genus *Origanum*: A Review. *Journal of Pharmacognosy and Phytotherapy*, **5**, 170-177.

[8] Han, F., Ma, G.Q., Yang, M., Yan, L., Xiong, W., Shu, J.C., Zhao, Z.D. and Xu, H.L. (2017) Chemical Composition and Antioxidant Activities of Essential Oils from Different Parts of the Oregano. *Journal of Zhejiang University-Science B*, **18**, 79-84. https://doi.org/10.1631/jzus.B1600377

[9] Yan, F., Azizi, A., Janke, S., Schwarz, M., Zeller, S. and Honermeier, B. (2016) Antioxidant Capacity Variation in the Oregano (*Origanum vulgare* L.) Collection of the German National Genebank. *Industrial Crops and Products*, **92**, 19-25. https://doi.org/10.1016/j.indcrop.2016.07.038

[10] Akrayi, H.F.S., Salih, R.M.H. and Hamad, P.A. (2015) *In Vitro* Screening of Antibacterial Properties of *Rhus coriaria* and *Origanum vulgare* against Some Pathogenic Bacteria. *ARO-The Scientific Journal of Koya University*. **3**, 35-41.

https://doi.org/10.14500/aro.10085

[11] Goncearіuc, M., Balmuş, Z., Benea, A., Barsan, V. and Sandu T. (2015) Biochemical Diversity of the *Origanum vulgare* L. ssp. *vulgare* and *Origanum vulgare* ssp. Hirtum (Link) Iestswaart Genotypes from Moldova. *Journal of ASM Life Sciences*, No. 2, 92-100.

[12] Zhang, X.L., Guo, Y.S., Wang, C.H., Li, G.Q., Xu, J.J., Chung, H.Y., Ye, W.C., Li, Y.L and Wang G.C. (2014) Phenolic Compounds from *Origanum vulgare* and Their Antioxidant and Antiviral Activities. *Food Chemistry*, **152**, 300-306. https://doi.org/10.1016/j.foodchem.2013.11.153

[13] Gîrd, C.E., Duțu, L.E., Costea, T., Nencu, I., Popescu, M. and Olaru, O. (2016) Preliminary Research Concerning the Obtaining of Herbal Extracts with Potential Neuroprotective Activity Note I. Obtaining and Characterization of a Selective *Origanum vulgare* L. Dry Extract. *Farmacia*. **64**, 680-687

[14] Sarrou, E., Tsivelika, N., Chatzopoulou, P. and Mavromatis, A. (2016) P16: Evaluation of Agronomical and Qualitative Characteristics of Greek Oregano (*Origanum vulgare* ssp. *hirtum*) Germplasm for Breeding Purposes. *6th International Symposium Breeding Research on Medicinal and Aromatic Plants,* BREEDMAP 6, Quedlinburg, 19-23 June 2016, 139-142.

[15] Leyva-López, N., Gutiérrez-Grijalva, E.P., Vazquez-Olivo, G. and Heredia, J.B. (2017) Essential oils of Oregano: Biological Activity beyond Their Antimicrobial Properties. *Molecules*, **22**, Article No. 989. https://doi.org/10.3390/molecules22060989

[16] Goleniowski, M.E., Flamarique, C. and Bima, P. (2003) Micropropagation of Oregano (*Origanum vulgare*×*applii*) from Meristem Tips. *In Vitro Cellular & Developmental Biology—Plant*, **39**, 125-128. https://doi.org/10.1079/IVP2002361

[17] Murashige, T. and Skoog, F. (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum,* **15**, 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

[18] Shah, R.R. and Dalal, K.C. (1980) *In Vitro* Multiplication of *Glycyrrhiza*. *Current Science India*, **49**, 69-71.

[19] Badoc, A. (1982) Contribution à l'étude des phénomènes d'organogenèse et de callogenèse de tissus de Fenouil vulgaire (*Foeniculum vulgare* subsp. *capillaceum* var. *vulgare* (Mill.) Thellung) cultivés *in vitro*; Analyse des constituants des huiles essentielles des explants. Diplôme d'Etudes Approfondies, Université de Lille, Flandres Artois, 78 f.

[20] Margara, J. (1978). Mise au point d'une gamme de milieux minéraux pour les conditions de la culture "*in Vitro*". *Comptes Rendus de l'Académie d'Agriculture de France*, **64**, 654-661. https://gallica.bnf.fr/ark:/12148/bpt6k6243937v/f666.item

[21] Gamborg, O.L., Miller, R.A. and Ojima, K. (1968) Nutrient Requirements of Suspension Cultures of Soybean Root Cells. *Experimental Cell Research*, **50**, 151-158. https://doi.org/10.1016/0014-4827(68)90403-5

[22] Schenk, R.U. and Hildebrandt, A.C. (1972). Medium and Techniques for Induction and Growth of Monocotyledonous and Dicotyledonous Plant Cell Cultures. *Canadian Journal of Botany*, **50**, 199-204. https://doi.org/10.1139/b72-026

[23] Shetty, K., Curtis, O.F., Levin, R.E., Witkowsky, R. and Ang, W. (1995) Prevention of Vitrification Associated with *in Vitro* Shoot Culture of Oregano. (*Origanum vulgare*) by *Pseudomonas* spp. *Journal of Plant Physiology*, **147**, 447-451. https://doi.org/10.1016/S0176-1617(11)82181-4

[24] Cristea Tina, O., Fălticeanu, M. and Prisecaru, M. (2008) Considerations Regarding

the Effects of Growth Regulators over the "*in Vitro*" Morphogenetic Reaction at *Origanum vulgare* L. *Journal of Plant Development*, **15**, 133-128.

[25] El Beyrouthy M., Elian G., Abou Jaoudeh, C. and Chalak, L. (2015) *In Vitro* Propagation of O*riganum syriacum* and *Origanum ehrenbergii*. *Acta Horticulturae*, **1083**, 169-172. https://doi.org/10.17660/ActaHortic.2015.1083.19

[26] Nicuţă, D. and Lazar, I. (2018) Studies on the Morphogenetic Reaction of *Origanum vulgare* L. Explants *in Vitro* Cultivation. *Studii şi Cercetări*, **27**, 63-66.

[27] Pandey, A., Belwal, T., Tamta, S., Bhatt, I.D. and Rawal, R.S. (2019) Phenolic Compounds, Antioxidant Capacity and Antimutagenic Activity in Different Growth Stages of *in Vitro* Raised Plants of *Origanum vulgare* L. *Molecular Biology Reports*, **46**, 2231-2241. https://doi.org/10.1007/s11033-019-04678-x

[28] Fokina, A., Denysiuk, K. and Satarova, T. (2020) Ризогенез живців *Origanum vulgare* L. при мікроклональному розмноженні *in vitro*. [*Origanum vulgare* L. Cuttings Rhizogenesis in Microclonal Reproduction *in Vitro*]. *Innovative Biosystems and Bioengineering*, **4**, 51-63. https://doi.org/10.20535/ibb.2020.4.1.192191

[29] Morone-Fortunato, I. and Avato, P. (2008) Plant Development and Synthesis of Essential Oils in Micropropagated and Mycorrhiza Inoculated Plants of *Origanum vulgare* L. ssp. *hirtum* (Link) Ietswaart. *Plant Cell, Tissue and Organ Culture*, **93**, Article No. 139. https://doi.org/10.1007/s11240-008-9353-5

[30] Economou, G., Panagopoulos, G., Tarantilis, P., Kalivas, D., Kotoulas, V., Travlos, I.S., Polysiou, M. and Karamanos, A. (2011) Variability in Essential Oil Content and Composition of *Origanum hirtum* L., *Origanum onites* L., *Coridothymus capitatus* (L.) and *Satureja thymbra* L. Populations from the Greek Island Ikaria. *Industrial Crops and Products*, **33**, 236-241. https://doi.org/10.1016/j.indcrop.2010.10.021

[31] Korkor, A.M., Mohamed, S.A., El-Kafie, O.M.A. and Gohar, A.A. (2017) Adaptation of the *in Vitro* Culture of *Origanum majorana* L. for Production of Phenolic Acids. *IOSR Journal of Pharmacy and Biological Sciences*, **12**, 30-38. https://doi.org/10.9790/3008-1202013038

[32] Abdallah, S.A.S., Yakoup, M.Y.A. and Abdalla, M.Y.A. (2017) Micropropagation of Oregano (*Origanum syriacum* L.) through Tissue Culture Technique. *Journal of Plant Production*, **8**, 635-639. https://doi.org/10.21608/jpp.2017.40497 https://jpp.journals.ekb.eg/article_40497_104d284776c5ba304a849e52b14bd263.pdf

[33] Zayova, E.G., Geneva, M.P., Miladinova-Georgieva, K.D., Hristozkova, M.G. and Stancheva, I.V. (2019) Impact of Plant Growth Regulators on Greek Oregano Micropropagation and Antioxidant Activity. *Biosciences*, *Biotechnology Research Asia*, **16**, 297-305. https://doi.org/10.13005/bbra/2746

[34] Nanova, Z. and Slavova, Y. (2006) Mass Vegetative Propagation of Winter Marjoram (*Origanum Vulgare* ssp. *Hirtum* (Link) Jetswaart). *Bulgarian Journal of Agricultural Science*, **12**, 531-536.

[35] El Ansari, Z.N., Boussaoudi, I., Benkaddour, R., Tahiri, H., El Oualkadi, A., Badoc, A., Martin, P. and Lamarti, A. (2020) Micropropagation of the Moroccan endemic Plant & *Thymus broussonetii* Boiss. with Aromatic-Medicinal Value and Conservation Concern. *American Journal of Plant Sciences*, **11**, 913-938. https://doi.org/10.4236/ajps.2020.116067

[36] Sarropoulou, V. and Maloupa, E. (2019) Effect of Polyamine Type and Concentration on *in Vitro* Propagation and *ex Situ* Conservation of *Sideritis raeseri* Boiss & Heldr. subsp. *raeseri*. *Research Journal of Life Sciences*, *Bioinformatics*, *Pharmaceutical and Chemical Sciences*, **5**, 583-598.

[37] Erland, L.A.E. and Mahmoud, S.S. (2014) Enhancing the Regeneration Efficiency of

Lavandin (*Lavandula* x *intermedia* cv Grosso): Effects of Light Quality, Medium Strength, Phenolic Control Agents, and Polyamines. University of British Columbia, Vancouver.

[38] Carlos Sánchez-Gras, M. and Segura, J. (1988) Morphogenesis *in Vitro* of *Sideritis angustifolia*: Effects of Auxins, Benzyladenine and Spermidine. *Plant Science*, **57**, 151-158. https://doi.org/10.1016/0168-9452(88)90081-7

[39] de Oliveira, R.C., Asmar, S.A., Silva, H.F. de J., Morais, T.P. de M. and Luz, J.M.Q. (2019) Regulators, Culture Media and Types of Lights *in Vitro* Lavender Culture. *Ciência Rural*, **49**, Article ID: e20180966, 7 p. https://doi.org/10.1590/0103-8478cr20180966

[40] Frabetti, M., Gutiérrez-Pesce, P., Mendoza-de Gyves, E. and Rugini, E. (2009) Micropropagation of *Teucrium fruticans* L., an Ornamental and Medicinal Plant. *In Vitro Cellular & Developmental Biology—Plant*, **45**, 129-134. https://doi.org/10.1007/s11627-009-9192-z

[41] Fazal, H., Shinwari, Z.K., Ahmad, N. and Abbasi, B.H. (2016) Factors Influencing *in Vitro* Seed Germination, Morphogenetic Potential and Correlation of Secondary Metabolism with Tissue Development in *Prunella vulgaris* L. *Pakistan Journal of Botany*, **48**, 193-200.

[42] Harfi, B., Benahmed, A. and Karkour, L. (2019) Characterization of *Origanum glandulosum* Desf. Essential Oils Collected from Different Culture Conditions towards Standardized *ex Situ* production. *Journal of Essential Oil Bearing Plants*, **22**, 838-850. https://doi.org/10.1080/0972060X.2019.1646163

[43] Sevindik, B., İzgü, T, Şimşek, Ö., Tütüncü, M., Cürük, P., Yilmaz, Ö., Kaynak, G., Aka Kaçar, Y., Teixeira da Silva, J.A. and Mendi, Y.Y. (2017) *In Vitro* Culture of Turkish *Origanum sipyleum* L. *American Journal of Plant Biology*, **2**, 32-36.

[44] Arumugam, G., Sinniah, U.R., Swamy, M.K. and Lynch, P.T. (2020) Micropropagation and Essential Oil Characterization of *Plectranthus amboinicus* (Lour.) Sprengel, an Aromatic Medicinal Plant. *In Vitro Cellular & Developmental Biology—Plant*, **56**, 491-503. https://doi.org/10.1007/s11627-020-10056-1

[45] Alkowni, R., Solyman, E. and Abu Qauod, H. (2017) Introducing Some of Threatened *Thymus* Species to *in Vitro* Tissue Culturing as an Approach for Their Conservation. *Pakistan Journal of Botany*, **49**, 259-264.

[46] Ozudogru, E.A., Kaya, E., Kirdok, E. and Issever-Ozturk, S. (2011) *In Vitro* Propagation from Young and Mature Explants of Thyme (*Thymus vulgaris* and *T. longicaulis*) Resulting in Genetically Stable Shoots. *In Vitro Cellular & Developmental Biology—Plant*, **47**, 309-320. https://doi.org/10.1007/s11627-011-9347-6

[47] Sevindik, B., Tütüncü, M., İzgü, T., Yilmaz, Ö., Kaynak G., Çürük, P. and Mendi, Y.Y. (2020) Efficient Micropropagation Protocol for *Lamium garganicum* L. subsp. *striatum* (Sm.) Hayek var. *striatum* Grown Naturally in Turkey. *Cukurova Journal of Agricultural and Food Sciences*, **35**, 89-98. https://doi.org/10.36846/CJAFS.2020.22