Scientific Research Publishing

American Journal of Plant Sciences, 2022, 13, 1126-1144
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

Interaction Effect of Plant Growth Regulators on Shoot Micropropagation of Aromatic Plant *Origanum elongatum* (Bonnet) Emberger & Maire

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How to cite this paper: Benkaddour, R., Ali, N.B., Azaroual, L., Martin, P. and Lamarti, A. (2022) Interaction Effect of Plant Growth Regulators on Shoot Micropropagation of Aromatic Plant *Origanum elongatum* (Bonnet) Emberger & Maire. *American Journal of Plant Sciences*, **13**, 1126-1144.
https://doi.org/10.4236/ajps.2022.138076

Received: May 14, 2022
Accepted: August 22, 2022
Published: August 25, 2022

Abstract

Origanum elongatum (Bonnet) Emb. & Maire, is a medicinal, aromatic and endemic plant of Morocco, characterized by its pharmacological effects, and is commonly used for the production of essential oils and aromas, resulting in high harvest and overexploitation pressure. This is why the present study aims to implement the *in vitro* micropropagation of *Origanum elongatum* for optimal vitroplant production. Six macroelements were tested (SH, SD, N30K, MS, MSm and B5) and the SD medium was selected for vegetative propagation of the explants. Seven cytokinins: adenine (Ad), N6-(2-Isopentenyl) adenine, zeatin (Zeat), kinetin (Kin), benzyladenine (BAP), 1,3-diphenylurea (DPU) and thidiazuron (TDZ) were then evaluated at five concentrations (0.44, 1.33, 2.22, 3.11 and 4.44 µM/L) on growth, development, budding, rooting and hyperhydricity. 0.44 µM Kin was selected and combined with three auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (AIB), and 1-naphthaleneacetic acid (NAA) at four concentrations (1.14, 2.85, 4.56 and 6.27 µM/L) to improve rooting and association with 1.14 µM IAA was shown to be efficient for roots development. Different concentrations of gibberellic acid (0.29, 1.5, 2.60 and 2.89 µM/L), combined with 0.44 µM/L Kin and 1.14 µM/L IAA, were tested and 2.60 µM/L GA_3 gave maximum buds and shoots. Then, the combination of three polyamines at five concentrations (1.134, 3.402, 5.67, 7.938 and 11.34 µM/L) with 0.44 µM Kin and 1.14 µM/L IAA showed an increase in the number of buds and shoots for 7.938 µM/L putrescine and 3.402 µM/L spermine. Finally, seedlings with good foliar and root development were acclimatized.

Keywords

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

1. Introduction

Origanum elongatum (Bonnet) Emb. & Maire is a Lamiaceae cladistically close to *Origanum grosii*, *O. compactum* and *O. vulgare* subsp. *virens* [1].

It is an aromatic species of limited geographical distribution in northeastern Morocco and extends from the Middle Atlas to the Rif mountain ranges, mainly at high altitude on the mountains) [2]. It grows in forests, rocks and matorals (thicker than scrub), more on siliceous substrates and deep, well-drained soils [3]. It is characterized by a fairly high bioclimatic plasticity ranging from semi-arid to wet per, with the thermo-Mediterranean and meso-Mediterranean vegetation stages being the most favorable [4]. In Morocco, its common Arabic name is Zaatar [3].

It is a 90 cm tall subshrub with light or dark upright stems, has glabrescent leaves, more or less glaucous, and a simple panicle of verticillasters about 40 mm long and 3 mm wide) [5]. It flowers from June to October [3]. White inflorescences are attached to vertical stems [6]. The abundance of inflorescences gives the species an ornamental interest [3]. Harvesting is possible in the first year but with a low yield of dry matter [7]. Germination is conditioned by several abiotic factors: a temperature of 20˚C, pH 6 and 1 g/L salinity are optimal for its germination [8]. Volatile compounds and extracts of *Origanum elongatum* exhibit antibacterial, antifungal, antiviral, antioxidant, vasodilator, anticorrosive and hepatoprotective effects [9]. *O. elongatum* contains several classes of bioactive compounds, including terpenoids, hydrocarbons, flavonoids, and phenolic compounds [10] [11].

Origanum elongatum is used in Morocco as an antibacterial, antifungal, antiviral, antioxidant, condiment and for the preservation of local food products such as melted butter and olives ([12] [13] [14]). It is a plant commonly used in Morocco for the production of essential oil and flavorings [15], which causes its overexploitation, especially since Morocco is ranked twelfth world exporter of medicinal and aromatic plants and the species is endemic to Morocco. High harvest pressure makes the species vulnerable and leads to its inclusion in Morocco's national red list ([16] [17]). A rapid assessment of its vulnerability by [18] classified it as a species in urgent need of conservation, restoration and sustainable management. Plant tissue culture can provide agriculture and industry with the plants needed to meet growing global demand, and is a powerful means of conserving, protecting, and domesticating vulnerable species. In this context, the aim of the present study is to establish an efficient protocol for the *in vitro* culture of *O. elongatum* originating in the Taza region, by the axillary bud technique.

2. Material and Methods

2.1. Plant Material

The explants used in this study were obtained from the apex of 3 to 4 centimeters of young plantlets of *Origanum elongatum* (Bonnet) Emberger & Maire. aged four weeks preserved in the Laboratory of Plant Biotechnology.

2.2. Effect of Mineral Nutrients

The mediums on which we performed the test are MS [19], SD [20], modified MS (MSm) [21], N30K [22], B5 [23] and SH [24], all of them were added with MS micronutrients and vitamins and 3% sucrose. The macronutrients gave the best results in terms of being served for all the following tests.

2.3. Effect of Cytokinins

Four cytokinins: Kin (kinetin), Zeat (zeatin), BAP (6-benzylaminopurine) and 2IP (2-isopentenyladenine) 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. Cytokinin 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 concentrations (1.14, 2.85, 4.56 and 6.27 µM/L) were tested with the most appropriate cytokinin determined in the preceding test. The medium containing only cytokinin served as a double control.

2.5. Effect of Cytokinins and Auxins Combined with Gibberellic Acid

Four concentrations of gibberellic acid (0.29, 1.5, 2.60 and 2.89 µM/L) were tested with the best combination of cytokinin and auxin. The medium containing 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 four 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 containing only cytokinin served as the control medium number 1 and the medium supplemented with the best combination of cytokinin and auxin served as double control.

2.7. Culture Conditions

The tubes were hermetically wrapped with aluminum foil and autoclaved 21 mn at 121˚C and 1 bar pressure. The cultures were incubated under specific con-

ditions (photoperiod: 18/6 h 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 plastic 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 three weeks, the surviving ones were transferred to large pots. Afterwards, they were placed under 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 days of 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 $p \leq 0.05$ are considered significant.

3. Results

3.1. Effect of Macronutrients

The maximum number of buds is marked in the case of the $N_{30}K$ medium (20.27), followed by the SD medium (19.31) and Mm medium (17.17), while the minimum number is noted in the case of the B5 medium (15.37). In addition, maximum shoot proliferation is reported for medium B5 (1.62) followed by Mm (1.53) and Ms (1.44); on the other hand, it is minimal in the case of the SH medium (1.26) (**Table 1**, **Figure 1**).

Furthermore, root multiplication was at its maximum for SD medium (7.81), followed by MSm medium (5.64) and $N_{30}K$ medium (4.91) and at its minimum for MS and B5 medium (3.00). For the elongation of the stem, SD medium gave

better results (2.13 cm), followed by Mm (2.08) and $N_{30}K$ (2.00) and the shortest explants were generated in the B5 medium (Table 1, Figure 1).

Table 1. Effect of six macronutrients on the micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire.

Medium	Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
MS	100.00	1.62 ± 0.09^{b}	16.11 ± 0.97^{ab}	1.44 ± 0.10^{a}	94.44	3.00 ± 0.70^{b}
MS_m	77.77	2.08 ± 0.12^{ab}	17.17 ± 1.13^{ab}	1.53 ± 0.13^{a}	100.00	5.64 ± 0.41^{ab}
$N_{30}K$	88.88	2.00 ± 0.13^{ab}	20.27 ± 5.97^{ab}	1.33 ± 0.58^{a}	80.56	4.91 ± 0.68^{b}
SH	83.33	1.85 ± 0.57^{ab}	16.53 ± 0.84^{ab}	1.26 ± 0.44^{a}	80.00	4.50 ± 0.68^{b}
SD	100.00	2.13 ± 0.11^{a}	19.31 ± 1.23^{a}	1.40 ± 0.10^{a}	93.75	7.81 ± 0.78^{a}
B_5	44.44	0.93 ± 0.07^{c}	15.37 ± 1.51^{c}	1.62 ± 0.15^{a}	75.00	3.00 ± 2.80^{b}

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

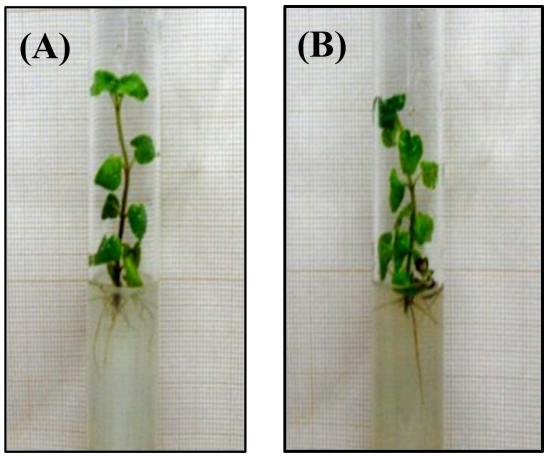

Figure 1. Effect on macronutrients on the micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire. (A) MS (B) SD.

In the other hand, the SD medium allowed total regeneration of the explants, followed by $N_{30}K$ (88.90%) and SH (80.55), while B5 gave a minimum percentage of regeneration (44.44). The maximum rate of rhizogenesis is marked in MSm medium (88.90%), followed by MS (94.44) and SD (93.75) and the minimum rate is mentioned for B5 (75.00) (Table 1). The absence of hyperhydric explants is marked in the six culture media (Table 1).

In conclusion, the SD medium is the best for the development and growth of vitroplants of *Origanum elongatum* and allows a total regeneration of explants. It has been selected for the following experiments.

3.2. Effect of Cytokinins

The addition of cytokinins to the SD medium gave better results. In terms of bud proliferation, it is maximal in the presence of 4.44 µM BAP (27.92), followed by 1.33 and 0.44 µM Kin (25.31 and 25.14 respectively). The minimum value is

given for 1.33 µM Adenine (13.02). In addition, the integration of cytokinins showed a positive effect on shoot multiplication; BAP at 3.11 µM gave the best result (2.14), followed by 4.44 µM BAP and 0.44 µM DPU (2.04 and 2.03 respectively) while the minimum value is given in the case of 4.44 µM DPU (1.11). In addition, an improvement in the number of roots is marked in the case of 4.44 µM 2IP (8.00), followed by 4.44 µM Zeat (7.66) and 2.22 µM 2iP (7.63). In contrast, the addition of 2.22 µM TDZ allowed minimal root proliferation (1.14). Moreover, the longest explants were regenerated in the presence of 0.44 µM Kin (3.91 cm), followed by 3.11 µM DPU (2.76) and 2.22 µM Kin (2.74 cm). However, shorter explants are obtained are obtained by the DPU at 4.44 µM (0.90 cm) (Table 2, Figure 2).

Table 2. Effect of cytokinins on micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire.

Cytokinins (µM/L)		Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
Control (SD)		86.11	2.55 ± 0.27^{ab}	22.70 ± 1.80^{ab}	1.51 ± 0.09^{abc}	90.00	4.64 ± 0.55^{cde}
	0.44	72.22	1.86 ± 0.11^{bcde}	14.30 ± 1.07^{b}	1.57 ± 0.09^{abc}	69.23	3.26 ± 0.55^{d}
	1.33	41.66	2.12 ± 0.10^{bcd}	13.02 ± 0.74^{b}	1.42 ± 0.11^{bc}	85.71	4.45 ± 0.59^{cde}
Ad	2.22	72.22	1.73 ± 0.08^{cd}	14.75 ± 0.91^{ab}	1.62 ± 0.09^{abc}	58.62	2.72 ± 0.49^{d}
	3.11	66.66	1.83 ± 0.07^{cd}	15.00 ± 1.12^{ab}	1.54 ± 0.12^{abc}	86.36	5.04 ± 0.74^{abcde}
	4.44	75.00	2.12 ± 0.14^{bcd}	15.00 ± 0.99^{ab}	1.86 ± 0.49^{abc}	50.00	1.86 ± 0.49^{d}
	0.44	83.33	1.58 ± 0.10^{ef}	20.66 ± 1.23^{ab}	1.50 ± 0.09^{bc}	100.00	6.96 ± 0.50^{abcd}
	1.33	72.22	1.29 ± 0.11^{ef}	$22,30 \pm 1.24^{ab}$	1.76 ± 0.08^{abc}	100.00	6.76 ± 0.51^{abcde}
2ip	2.22	91.67	1.72 ± 0.09^{ef}	$23,45 \pm 1.12^{ab}$	1.50 ± 0.09^{bc}	100.00	7.63 ± 0.38^{a}
	3.11	88.90	1.75 ± 0.10^{cdef}	$24,00 \pm 1.15^{ab}$	1.71 ± 0.10^{abc}	93.75	7.12 ± 0.67^{abcd}
	4.44	61.11	0.90 ± 0.06^{f}	18.90 ± 1.40^{ab}	1.81 ± 0.10^{abc}	100.00	8.00 ± 0.36^{a}
	0.44	86.11	1.46 ± 0.08^{ef}	20.66 ± 1.23^{ab}	1.50 ± 0.09^{bc}	100.00	6.96 ± 0.50^{abcd}
	1.33	83.33	1.67 ± 0.07^{ef}	22.30 ± 1.24^{ab}	1.76 ± 0.08^{abc}	100.00	1.05 ± 0.06^{abcd}
Zeat	2.22	80.56	1.42 ± 0.10^{ef}	23.45 ± 1.12^{ab}	1.50 ± 0.09^{bc}	100.00	1.05 ± 0.06^{abc}
	3.11	52.78	1.24 ± 0.08^{ef}	24.00 ± 1.15^{ab}	1.71 ± 0.10^{abc}	100.00	1.11 ± 0.09^{ab}
	4.44	50.00	1.32 ± 0.07^{ef}	18.90 ± 1.40^{ab}	1.81 ± 0.10^{abc}	100.00	1.07 ± 0.05^{abcd}
	0.44	97.22	3.91 ± 0.39^{a}	25.14 ± 1.41^{ab}	1.54 ± 0.12^{abc}	71.42	4.17 ± 0.58^{e}
	1.33	80.55	2.53 ± 0.27^{bcd}	25.31 ± 1.93^{ab}	1.65 ± 0.15^{abc}	89.65	5.34 ± 0.54^{abcde}
Kin	2.22	80.56	2.74 ± 0.28^{b}	20.13 ± 1.18^{ab}	1.34 ± 0.08^{c}	82.76	4.62 ± 0.63^{cde}
	3.11	75.00	1.85 ± 0.16^{bcdef}	20.51 ± 0.98^{ab}	1.81 ± 0.11^{abc}	88.90	4.70 ± 0.67^{bcde}
	4.44	75.00	1.91 ± 0.13^{bcde}	19.25 ± 1.13^{ab}	1.55 ± 0.09^{abc}	92.60	5.37 ± 0.67^{abc}
	0.44	86.11	1.70 ± 0.13^{cdef}	18.91 ± 1.39^{ab}	1.58 ± 0.13^{abc}	90.00	4.41 ± 0.59^{de}
	1.33	72.22	1.46 ± 0.10^{ef}	19.33 ± 0.99^{ab}	1.70 ± 0.11^{abc}	69.23	4.54 ± 0.73^{cde}
BAP	2.22	41.66	1.35 ± 0.09^{ef}	21.06 ± 1.44^{ab}	1.62 ± 0.10^{abc}	85.71	5.00 ± 0.64^{abcde}
	3.11	72.22	1.32 ± 0.06^{ef}	22.70 ± 1.48^{ab}	2.14 ± 0.30^{ab}	58.62	5.20 ± 0.61^{abcde}
	4.44	66.66	1.28 ± 0.06^{ef}	27.92 ± 1.89^{a}	2.03 ± 0.14^{ab}	86.36	5.34 ± 0.71^{abcde}

Continued

	0.44	75.00	2.54 ± 0.18^{ab}	21.92 ± 0.18^{ab}	2.03 ± 0.10^{ab}	50.00	3.92 ± 0.76^{d}
	1.33	75.00	2.16 ± 0.15^{bcd}	18.22 ± 1.04^{ab}	1.70 ± 0.08^{abc}	62.96	3.33 ± 0.64^{e}
DPU	2.22	61.11	2.20 ± 0.19^{abc}	16.72 ± 1.18^{ab}	1.59 ± 0.10^{abc}	68.18	4.59 ± 0.73^{cde}
	3.11	80.55	2.76 ± 0.20^{ab}	17.93 ± 1.08^{ab}	1.68 ± 0.14^{abc}	93.10	6.27 ± 0.74^{abcde}
	4.44	72.22	2.21 ± 0.13^{abc}	16.61 ± 0.65^{ab}	1.11 ± 0.06^{c}	80.76	5.15 ± 0.70^{abcde}
	0.44	80.55	2.17 ± 0.11^{abc}	20.68 ± 1.24^{ab}	1.75 ± 0.11^{abc}	75.86	4.31 ± 0.63^{cde}
	1.33	75.00	2.00 ± 0.14^{bc}	20.51 ± 1.38^{ab}	1.70 ± 0.11^{abc}	48.14	2.70 ± 0.64^{d}
TDZ	2.22	58.33	1.71 ± 0.10^{c}	16.76 ± 1.32^{ab}	1.48 ± 0.11^{bc}	28.57	1.14 ± 0.44^{e}
	3.11	41.66	1.94 ± 0.12^{bc}	16.88 ± 1.14^{ab}	1.68 ± 0.11^{ab}	24.00	1.20 ± 0.54^{e}
	4.44	66.66	1.78 ± 0.13^{c}	19.25 ± 1.14^{ab}	1.62 ± 0.10^{abc}	54.16	1.54 ± 0.34^{e}

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

Figure 2. Effect of cytokinins on micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire. (A) Control; (B) 0.44 µM Ad; (C) 3.11 µM 2ip MS; (D) 3.11 µM Zeat; (E) 0.44 µM Kin; (F) 4.44 µM BAP; (G) 4.44 µM DPU; (H) 0.44 µM TDZ.

To sum up, the integration of cytokinins into the culture medium proves advantageous for the development of explants and more particularly the aerial part. 0.44 µM Kin was chosen for the following experiments because it gave good results in terms of elongation of the vitro plants and multiplication of shoots and buds. In addition, it gave a maximum rate of regeneration with no hyperhydric plants. The root part will be improved by combining 0.44 µM Kin with three auxins at increasing concentrations.

3.3. Effect of Cytokinins Combined with Auxins

The combination of 0.44 µM Kin and the three auxins shows a favorable effect on the growth of *Origanum elongatum* explants.

Thus, the medium added with 1.14 µM of IAA generates the maximum number of buds (28.26), followed by 6.27 µM of IAA (26.52) and 4.56 µM of NAA (25.4). In contrast, the medium supplemented with 1.14 µM NAA provides a minimum number of buds (17.42). For shoot proliferation, it is highest in the presence of 1.14 µM IAA (1.97), followed by 6.27 µM IAA (1.88) and 4.56 µM NAA (1.86). The IBA at 1.14 µM records the lowest value (1.40) (Table 3, Figure 3). In addition, the combination of 1.14 µM IBA and 1.14 µM IAA respectively with 0.44 µM Kin provides best results in terms of explant elongation (4.34 and

4.06 cm), followed by 4.56 µM NAA (3.95 cm), while the shortest explants is obtained in the presence of 4.56 NAA (2.34 cm) (Table 3, Figure 3).

Table 3. Effect of auxins combined with 0.44 µM Kin on the micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire.

Auxins (µM/L)		Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
Control 1 SD		86.11	2.55 ± 0.27^{c}	22.70 ± 1.8^{abcc}	1.51 ± 0.09^{a}	90.00	4.64 ± 0.55^{bc}
Control 2 (0.44 µM Kin)		97.22	3.91 ± 0.39^{ab}	25.14 ± 1.41^{abc}	1.54 ± 0.12^{a}	71.42	4.17 ± 0.58^{c}
IAA	1.14	86.11	4.06 ± 0.37^{ab}	28.26 ± 1.73^{a}	1.96 ± 0.21^{a}	93.55	6.58 ± 0.66^{abc}
	2.85	91.66	3.87 ± 0.23^{ab}	23.39 ± 1.44^{abcd}	1.54 ± 0.12^{a}	100.00	6.78 ± 0.55^{abc}
	4.56	91.66	3.95 ± 0.27^{ab}	23.57 ± 1.41^{abcd}	1.42 ± 0.08^{a}	93.93	6.90 ± 0.63^{abc}
	6.27	94.44	3.56 ± 0.29^{abc}	26.52 ± 1.55^{ab}	1.88 ± 0.11^{a}	91.17	6.47 ± 0.65^{abc}
IBA	1.14	91.66	4.34 ± 0.26^{a}	18.78 ± 0.90^{cd}	1.39 ± 0.08^{a}	100.00	7.66 ± 0.50^{ab}
	2.85	86.11	3.37 ± 0.25^{abc}	20.00 ± 1.24^{abcd}	1.61 ± 0.11^{a}	80.64	6.25 ± 0.90^{abc}
	4.56	66.70	3.38 ± 0.35^{abc}	23.08 ± 1.41^{abcd}	1.62 ± 0.10^{a}	83.33	5.41 ± 0.87^{abc}
	6.27	77.41	3.15 ± 0.25^{abc}	23.80 ± 1.65^{abcd}	1.61 ± 0.08^{a}	86.11	3.90 ± 0.64^{c}
NAA	1.14	86.11	3.67 ± 0.20^{abc}	17.41 ± 1.09^{c}	1.41 ± 0.09^{a}	100.00	8.67 ± 0.67^{a}
	2.85	69.44	3.42 ± 0.25^{abc}	19.92 ± 1.44^{bcd}	1.56 ± 0.10^{a}	88.00	6.60 ± 0.90^{abc}
	4.56	77.77	2.34 ± 0.15^{c}	25.42 ± 1.60^{abc}	1.85 ± 0.16^{a}	78.57	4.21 ± 0.57^{c}
	6.27	97.22	2.97 ± 0.16^{bc}	21.54 ± 1.56^{bcd}	1.57 ± 0.15^{a}	80.00	5.45 ± 0.61^{bc}

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

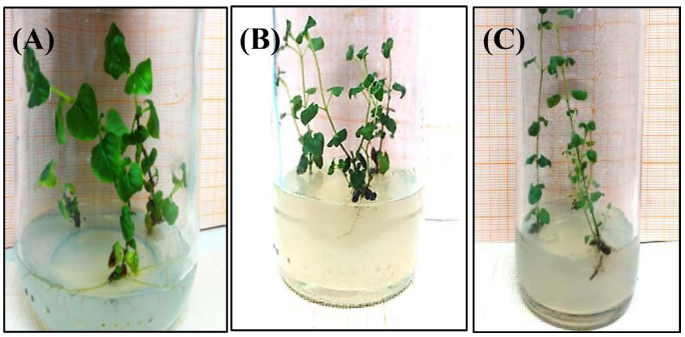

Figure 3. Effect of auxins combined with 0.44 µM Kin on micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire. (A) 1.14 µM IAA; (B) 4.56 µM NAA; (C) 6.27 µM NAA.

On the other hand, the addition of auxins to the culture medium improves the propagation of the roots. In fact, the maximum number of roots is recorded in the presence of 1.14 µM IAA (8.67), followed by 1.14 µM IBA (7.66) and 4.56 µM IAA (6.90) while the minimum number of roots is noted in the presence of 6.27 µM IBA (3.90) (Table 3, Figure 3).

The highest rate of regeneration is reported in case of 6.27 µM NAA (97.22%), followed by 6.27 µM IAA (94.44) and 4.56 µM IAA and 1.14 IBA µM with

91.67%. The highest percentage of rooted seedlings is observed with 1.14 µM NAA and 1.14 µM IBA (100%), followed by 4.56 µM IAA (93.93) and 6.27 µM IAA (91.17). Hyperhydricity is absent in all culture media (Table 3).

All in all, the combination of 0.44 µM Kin and 1.14 µM IAA is the most advantageous for the development of both parts of the plant. Also, it allows a relatively high rate of regeneration and rhizogenesis.

3.4. Effect of Cytokinins and Auxins Combined with Gibberellic Acid

Among the four concentrations of GA_3 combined at 0.44 µM Kin + 1.14 µM IAA, we find that the culture medium supplemented with 2.60 µM of GA_3 is the best in terms of bud multiplication (20.17), followed by the control medium 2 (0.44 µM Kin + 1.14 µM IAA) (18.35) and the medium supplemented with 1.15 µM GA_3 (18.00). However, control medium 2 regenerates a small number of buds (13,523). Moreover, there is no significant difference in the proliferation of the shoots, despite it has its maximum in the case of control medium 2 and in that supplemented with 2.60 µM GA_3 (2.05 and 2.00 respectively) and followed by 0.29 µM GA_3 (1.85); on the other hand, it is at its minimum in the case of 1.15 µM GA_3 (1.77). In addition, the root multiplication reaches its maximum in the presence of 0.29 µM GA_3 (5.70), followed by 2.80 µM GA_3 (3.45) and the control medium 2 (3.00). In return, the multiplication is minimal on the control medium 1 (SD only) (1.14) (Table 4, Figure 4). Also, the longest explants are regenerated on the culture medium supplemented with 0.29 µM GA_3 (3.83 cm), followed by the control medium 2 (2.23) and the medium containing 2.60 µM GA_3 What is more, the rate of rhizogenesis is maximal in the medium supplemented with 0.29 µM GA_3 (100%), followed by the control medium 2 (0.44 µM Kin + 1.14 µM IAA) (70.58) and the medium containing 2.80 µM GA_3 (66.66). The highest rate of regeneration was observed for 2.60 µM GA_3 (94.44%), followed by 2.80 µM GA_3 and 1.15 µM GA_3 (72.22). No cases of hyperhydria were observed for the different combinations tested (Table 4).

Table 4. Effect of gibberellic acid combined with 0.44 µM Kin and 1.14 µM IAA on the micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire.

GA_3 (µM/L)		Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
Control 1 (SD)		47.22	2.23 ± 0.27^{b}	18.35 ± 1.19^{ab}	2.05 ± 0.05^{a}	70.58	3.00 ± 0.59^{bc}
Control 2 (0.44 µM Kin+1.14 µM IAA)		58.33	1.92 ± 0.13^{b}	13.52 ± 0.74^{c}	1.80 ± 0.08^{a}	33.33	1.14 ± 0.42^{c}
GA_3	0.29	72.22	3.82 ± 0.20^{a}	14.61 ± 0.56^{bc}	1.84 ± 0.07^{a}	10.,00	5.96 ± 0.44^{a}
	1.5	61.11	1.91 ± 0.12^{b}	18.00 ± 0.91^{ab}	1.77 ± 0.09^{a}	40.90	1.50 ± 0.43^{c}
	2.60	94.44	2.30 ± 0.15^{b}	20.17 ± 1.04^{a}	2.00 ± 0.12^{a}	35.29	1.61 ± 0.42^{c}
	2.89	75.0	2.11 ± 0.11^{b}	16.51 ± 0.87^{bc}	1.81 ± 0.07^{a}	66.66	3.48 ± 0.55^{b}

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

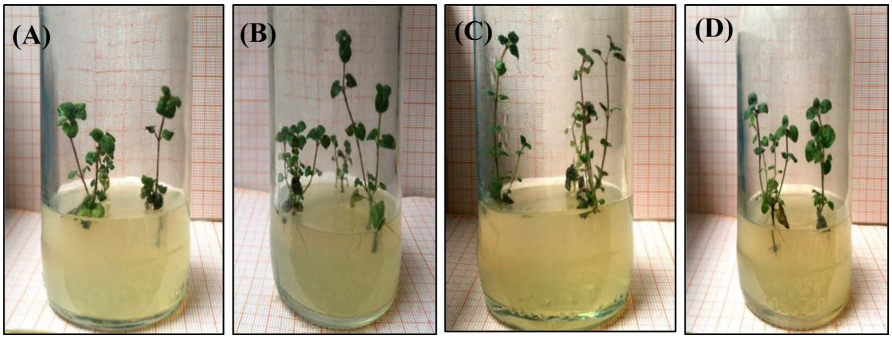

Figure 4. Effect of GA_3 combined with 0.44 µM Kin and 1.14 µM IAA on micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire. (A) 0.29 µM GA_3; (B) 1.15 µM GA_3; (C) 2.60 µM GA_3; (D) 2.89 µM GA.

(2.30) (**Table 4**, **Figure 4**).

3.5. Effect of Cytokinins and Auxins Combined with Polyamines

Integration of three polyamines at different concentrations with 0.44 µM Kin and 1.14 µM IAA resulted in some changes in the micropropagation of vitroplants of *Origanum elongatum.*

Thus, compared with the two control mediums, the maximum number of buds is marked in the case of 7.938 µM of Putrescine (18.44), followed by 5.67 and 1.134 µM of putrescine (18.40 and 18.00). Otherwise, the medium supplemented with 7.938 µM of spermine (14.20) regenerates a minimum number of buds. In terms of shoot multiplication, it is maximum in the case of 3.402 and 7.938 µM of spermine (1.86 and 1.85), followed by 1.134 µM putrescine (1.84). However, the control medium 1 gave the minimum value (1.55). In addition, the maximum number of roots is obtained in the case of 1.134 µM spermidine (4.909), followed by 7.938 and 5.67 µM putrescine (4.20 and 4.16, respectively), while the minimum number is recorded in the case of 3.402 µM Spermine (0.54) (**Table 5**, **Figure 5**).

Table 5. Effect of polyamines combined with 0.44 µM Kin and 1.14 µM IAA on the micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire.

Polyamines (µM/L)		Regeneration (%)	Shoot length (cm)	Number of buds	Number of shoots	Rooting (%)	Number of roots
(0.44 µM Kin)		72.22	3.66 ± 0.24^{ab}	16.13 ± 1.02^{a}	1.55 ± 0.10^{a}	41.37	2.03 ± 0.54^{bcd}
(0.44 µM Kin + 1.14 µM IAA)		80.55	4.20 ± 0.25^{a}	17.75 ± 0.96^{a}	1.73 ± 0.08^{a}	73.40	2.73 ± 0.40^{abcd}
Putrescine	1.134	72.22	3.53 ± 0.25^{ab}	18.00 ± 1.05^{a}	$1.84 \pm 0,13^{a}$	53.84	1.73 ± 0.45^{abc}
	3.402	80.55	3.66 ± 0.23^{ab}	17.44 ± 1.13^{a}	$1.79 \pm 0,07^{a}$	68.96	2.55 ± 0.48^{abcd}
	5.67	83.33	3.99 ± 0.34^{a}	18.40 ± 1.02^{a}	1.76 ± 0.10^{a}	80.00	4.16 ± 0.60^{abc}
	7.938	88.89	3.18 ± 0.23^{ab}	18.43 ± 1.09^{a}	1.81 ± 0.09^{a}	84.37	4.25 ± 0.56^{ab}
	11.34	86.11	3.63 ± 0.25^{ab}	18.38 ± 1.23^{a}	1.70 ± 0.09^{a}	80.64	3.87 ± 0.51^{abc}
Spermidine	1.134	91.67	3.68 ± 0.24^{ab}	17.09 ± 0.70^{a}	1.63 ± 0.08^{a}	81.98	4.90 ± 0.57^{a}

Continued

	3.402	77.77	2.77 ± 0.15^{b}	17.71 ± 0.78 ^{a}	1.85 ± 0.08^{a}	71.42	4.03 ± 0.78^{abc}
	5.67	88.89	3.49 ± 0.21^{ab}	17.50 ± 0.69^{a}	1.81 ± 0.07^{a}	71.87	3.59 ± 0.56^{abc}
	7.938	75.00	3.23 ± 0.19^{ab}	15.77 ± 0.71^{a}	1.69 ± 0.09^{a}	70.37	2.59 ± 0.48^{abcd}
	11.34	91.67	3.26 ± 0.19^{ab}	14.78 ± 0.60^{a}	1.63 ± 0.08^{a}	33.33	2.87 ± 0.40^{abcd}
	1.134	83.33	3.79 ± 0.22^{ab}	14.80 ± 0.58^{a}	1.66 ± 0.08^{a}	66.66	2.03 ± 0.45^{bcd}
	3.402	72.22	3.47 ± 0.22^{ab}	15.46 ± 0.74^{a}	1.69 ± 0.09^{a}	30.76	0.53 ± 0.19^{d}
Spermine	5.67	69.44	2,98 ± 0.27^{ab}	15.84 ± 0.95^{a}	1.72 ± 0.09^{a}	64.00	2.20 ± 0.48^{bcd}
	7.938	55.56	3.26 ± 0.27^{ab}	14.20 ± 0.92^{a}	1.85 ± 0.19^{a}	75.00	2.95 ± 0.51^{abcd}
	11.34	80.55	3.44 ± 0.33^{ab}	15.44 ± 0.74^{a}	1.47 ± 0.11^{ab}	78.78	0.82 ± 0.26^{d}

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

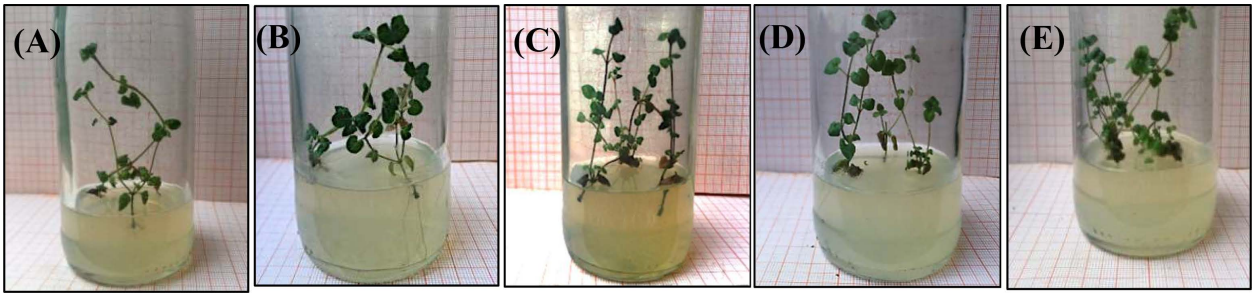

Figure 5. Effect of polyamines combined with 0.44 µM Kin and 1.14 µM IAA on the micropropagation of *Origanum elongatum* (Bonnet) Emb. & Maire. (A) 5.67 µM putrescine; (B) 3.402 µM psermidine; (C) 5.67 µM spermidine; (D) 3.402 µM spermidine; (E) 5.67 µM spermine.

Moreover, the elongation of the stem part reaches its maximum in the case of the control medium 2 (4.20 cm), followed by the medium supplemented with 5.67 and 11.34 µM putrescine (3.99 and 3.64 cm). However, it attains its minimum in the case of 3.402 µM spermidine (2.77 cm) (**Table 4**, **Figure 5**).

Additionally, the medium supplemented with 1.134 and 11.34 µM spermidine gave a high rate of regeneration (91.67%), followed by 7.938 µM putrescine and 5.67 µM spermidine (88.89%) and finally 11.34 µM of putrescine (86.11%). However, the minimum level is obtained in the medium supplemented with 7.938 µM spermine. The highest percentage of rhizogenesis is marked in the case of 7.938 µM putrescine (84.37%), followed by 11.34 and 5.67 µM putrescine (80.00%), while the lowest is reported in the case of 5.67 µM spermine (30.76%). In addition, the absence of hyperhydric explants is noted in the various combinations tested.

3.6. Acclimatization Plantlets

Vitroplants grown in medium supplemented with 0.44 µM Kin and 1.14 µM IAA show good root and foliar development. This medium allows a relatively high rate of regeneration and rhizogenesis with maximum root multiplication. For these reasons, it has been used for acclimatization. Seedlings from acclimatization show

good morphogenetic characteristic with 98% survival percentage (Figure 6).

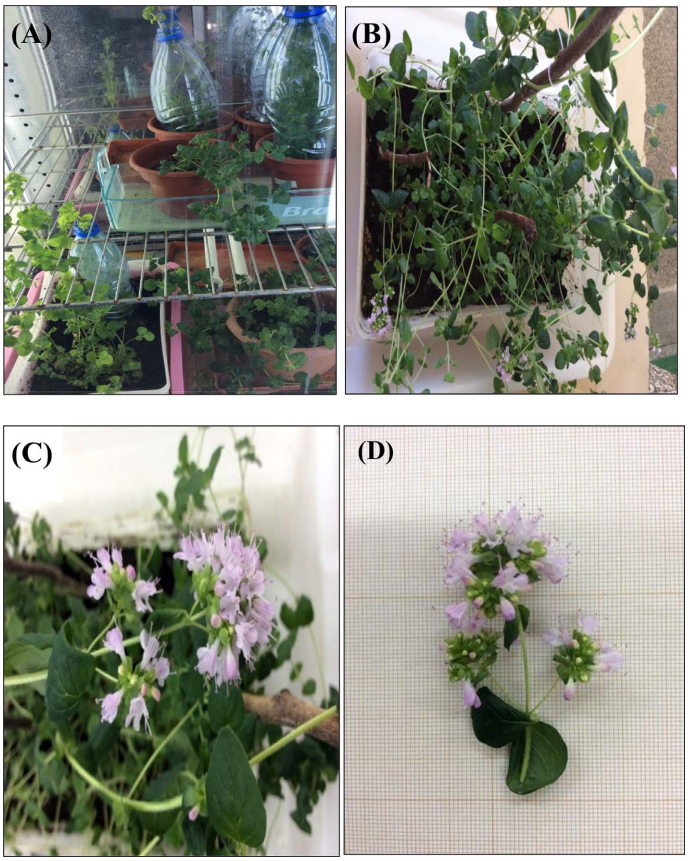

Figure 6. Acclimatization phase (A) after 3 months; (B) after 5 months; (C) after 7 months; (D) *Origanum elongatum* (Bonnet) Emb. & Maire inflorescences.

4. Discussion

The basic step in the micropropagation of plants is to choose the best culture medium that allows good development and growth of explants. For this reason, SD medium was selected for subsequent experiments because it offered good results in terms of bud and root multiplication and also the elongation of the two parts of the explants. In addition, it allows total regeneration of the vitroplants and the absence of hyperhydricity cases. However, in the majority of studies involving *in vitro* culture of oregano, MS medium was the most widely used [25]-[31]).

Thus, cytokinins are an important class of PGRs and their integration into the culture medium has led to changes in the micropropagation of *Origanum elongatum* Emb. & Mayor. In fact, the addition of 4.44 µM BAP to the culture medium made it possible to optimize the proliferation of buds. In addition, a maximum multiplication of shoots is indicated for 3.11 µM BAP. A high concentration of 2IP promoted the regeneration of a high number of roots. Moreover, a low concentration of Kin (0.44 µM) and a high concentration of DPU are shown to be efficient for elongation of the root part. Evaluation of cytokinin type and concentration was initiated in other species of the genus *Origanum*. Zayova

et al. (2016) [32] showed that 4.44 µM of Zeat induced optimal elongation, regeneration, and multiplication of shoots of *Origanum vulgare* L. subsp. *viridulum* (Martrin-Donos) Nyman (*Origanum heracleoticum* L.). In addition, Arafeh *et al.* (2003) [25] reported that the medium supplemented with 3.55 µM BAP favored regeneration of a maximum number of shoots and leaves and gave better elongation of vitroplants of *Origanum syriacum* L. Socorro *et al.* (1998) [33] evaluated the effect of cytokinin concentration; they found that 0.2 µM BAP was the best for the elongation of axillary stems, bud multiplication, and leaves of *Origanum vulgare* subsp. *virens* (Hoffmanns. & Link) Ietsw. (*Origanum bastetanum* Socorro, Arrebola & Espinar) vitroplants. However, Atar and Çölgeçen (2019) [26] reported that a high concentration of Kin (6.66 µM) was supportive of a high multiplication of shoots of *Origanum onites* L. Similarly, El Beyrouthy *et al.* (2015) [34] showed that the presence of an optimal concentration of BAP (8.88 µM) in the culture medium is shown to be efficient for regeneration and multiplication of vitroplants of *Origanum syriacum* L. and *O. ehrenbergii* Boiss. In addition, Kizil and Khawar (2017) [35] reported that the proliferation and regeneration of *Origanum acutidens* shoots was maximal in medium supplemented with 0.8 µM BAP. Moreover, the type and concentration of cytokinins were evaluated in other species of the Lamiaceae family ([36] [37] [38] [39]).

Integration of cytokinins into the culture medium led to improvements in the micropropagation of *Origanum elongatum* explants, in particular the Kin at 0.44 µM, as it gave better results in shoot elongation and multiplication and bud proliferation. In addition, it ensured complete regeneration of the explants and the absence of hyperhydria. Growth and development of the root part are optimized by combining 0.44 µM Kin with three auxins at increasing concentrations. In fact, in comparison with the two-control media, the root multiplication is improved by the combination of 0.44 µM Kin and 1.14 µM NAA. In addition, the combination of 0.44 µM Kin and 1.14 µM IAA contribute to the enhancement of root elongation. It also has a positive effect on the other parameters evaluated. Generally, the choice of the best cytokinin/auxin balance is very important for the good development of all parts of the plant, not only the root part. Thus, the best multiplication of the vitroplants of *Origanum vulgare* L. is indicated in the medium supplemented with 4.44 µM BAP and 4.92 µM IBA (Nicuță and Lazar, 2018 [40]). In addition, Cristea *et al.* (2008) [41] demonstrated that the combination of low concentration of BAP and 0.27 µM NAA is efficient for good proliferation of *Origanum vulgare* explants. Also, it favored the appearance and development of roots. Moreover, treatment with 0.28 µM BAP and 2.86 µM NAA was beneficial for the proliferation of all parts of vitroplants of 'Mendocino' oregano *Origanum* x *majoricum* Cambess [42]. In addition, Kizil and Khawar (2017) [35] demonstrated that combining a minimum concentration of BAP with 2.7 µM NAA was shown to be effective for the micropropagation of *Origanum acutidens* (Hand.-Mazz.) Ietswaart and allowed a total regeneration of acclimated plants. On the other hand, El Beyrouthy *et al.* (2015) [34] demonstrated that the

combination of 8.88 µM BAP and 0.054 µM NAA induced better responses in terms of multiplication, elongation and regeneration of shoots of *Origanum syriacum* and *O. ehrenbergii*. However, root growth and development are evident in MS medium lacking growth regulators. The search for the best cytokinin/auxin balance is reported in other Lamiaceae. Mozafari *et al.* (2015) [43] found that the combination between BAP and IBA or TDZ and 2,4-D induced callus formation, while IBA alone promoted root multiplication and elongation. Also, it allowed a maximum percentage rooting of *Satureja avromanica* Maroofi. Dode *et al.* (2003) [44] demonstrated that the presence of NAA in the culture medium inhibited root formation when combined with different concentrations of BAP (4.44 - 22.19 µM), while the greatest formation of *Ocimum basilicum* L. shoots occurred in the medium supplemented with 22.19 µM BAP and 1.08 µM ANA.

In addition, the combination of GA_3 at various concentrations with 0.44 µM Kin and 1.14 µM IAA brought some changes in the micropropagation of *Origanum elongatum* explants. Thus, in comparison with the control medium 2, the number of buds and shoots reaches its maximum values in the case of the medium supplemented with 2.60 µM of GA_3, while the number of roots has been maximum in the case of the medium supplemented with a minimum concentration of GA_3. Similarly, is observed for the elongation of the root and stem parts.

Investigations of the effect of GA_3 in combination with cytokinins and auxins on oregano development and growth *in vitro* have not been reported, but have been investigated in other Lamiaceae, such as *Salvia hispanica* L., whose highest percent regeneration and high shoot proliferation were reported in media supplemented with 0.75 or 1 µM BAP, 0.1 µM GA_3, and 0.1 µM NAA (Bueno *et al.* 2010) [45]. In *Lavandula dentata* L., the combination of 0.5 µM BAP, 0.3 µM GA_3, and 2.5 µM IBA allowed maximum shoot elongation and multiplication, whereas maximum regeneration was indicated for the medium supplemented with 10 µM BAP, 2.5 µM IBA, and 0.3 µM GA_3 [46]. Kousalya and Narmatha Bai (2016) [47] reported that the combination of cytokinins alone, including 2.22 µM BAP, 9.29 µM Kin and 2.88 µM GA_3, increased the multiplication and elongation of *Canscora alata* (Roth) Wall. (=*C. decussata* (Roxb.) Roem. & Schult.) and Shtereva *et al.* (2015) [48] demonstrated that the combination of 1.44 µM GA_3 with auxins, particularly 1.14 µM IAA and 9.84 µM IBA, favored better shoot and root elongation and increased propagation rate of *Sideritis scardica* Griseb. vitroplants. Additionally, in comparison with the two-control media, the addition of polyamines to the medium has a negative effect on the elongation of the stem part. On the other hand, high concentrations, especially of Putrescine and Spermidine, are favorable for elongation of the root part. The assessment of the effect of the combination of polyamines, cytokinins and auxins is not addressed in species of the genus *Origanum* or species of Lamiaceae. However, the effect of polyamines alone or in combination with either cytokinins or auxins is studied. In this context, stimulation of the regeneration of *Salvia officinalis* L. shoots *via* internode culture is demonstrated in the medium supplemented with

567 µM Putrescine and 2.27 µM TDZ [49] and the elongation and multiplication of the roots of *Tectona grandis* L.f. vitroplants was better in the medium supplemented with 18.08 mM putrescine and 2.46 µM IBA, in addition, the presence of putrescine accelerated shoot growth and development [50]. Application of polyamines alone, including spermine and spermidine at concentrations greater than 10 µM, inhibits the root formation of *Lavandula* × *intermedia* "Grosso" vitroplants (Erland and Mahmoud 2014) [51]. El Ansari *et al.* (2019) [36] found that the best propagation of buds, shoots and roots of *Thymus vulgaris* L. was marked in the medium supplemented with 10 µM spermine and demonstrated that 50 µM spermidine was effective for better propagation of roots whereas 10 µM of the same polyamine was favorable for root propagation.

5. Conclusions

This study is the first experiment of micropropagation of *Origanum elongatum*, endemic to Morocco.

The SD medium ensured good results in terms of bud and root multiplication and elongation of the stem and root parts while allowing total regeneration of the vitroplants and the absence of hyperhydricity. Kin at 0.44 µM has provided optimal results in term of bud proliferation and shoots elongation and multiplication; in addition, it afforded a total regeneration of vitroplants. Thus, the optimization of growth and development of the root part is ensured by the combination 0.44 µM Kin and 1.14 µM IAA.

Moreover, the combination of polyamines with 0.44 µM Kin and 1.14 µM IAA did not significantly improve the micropropagation of *Origanum elongatum*, particularly the proliferation of the culinary part. Certainly, a small increase in the number of buds and shoots is observed with 7.938 µM of putrescine and spermine. However, high concentrations of putrescine and spermidine favored better root elongation, and the combination of 2.60 µM GA_3 with 0.44 µM Kin and 1.14 µM IAA provided a maximum number of buds and shoots, and the medium supplemented with 1.14 µM GA_3 allowed a better root multiplication and elongation.

Finally, vitroplants from SD + 0.44 µM/L Kin/L + 1.14 µM/L IAA showing good foliar development and characterized by a persistent root system were selected for acclimatization and it was successfully carried out, in addition, a high survival rate after acclimatization was marked. As a result, the protocol followed in this study is efficient for the conservation, protection and domestication of this vulnerable species, in order to meet increasing market demand in a short time, however, the main disadvantage plant tissue culture through micropropagation methods is the relatively higher costs involved as compared to other methods, therefore recent findings on low-cost methods used in plant tissue culture for the *in vitro* propagation of plant are investigated.

Conflicts of Interest

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

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