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Effect of Explant Origin and Medium Plant Growth Regulators on In Vitro Shoot Proliferation and Rooting of *Salvia tomentosa,* a Native Sage of the Northeastern Mediterranean Basin

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Abstract: *Salvia tomentosa* is a perennial sage of the maquis vegetation, strongly aromatic, with medicinal and ornamental value. Aiming to facilitate the promotion of the species in the floriculture sector, its micropropagation was studied in terms of the effect of explant origin and medium plant growth regulators on in vitro cultures establishment, shoot proliferation and rooting. Shoot tip or single node explants excised from either in vitro grown seedlings or greenhouse plants grown from cuttings of mature plants were cultured on MS medium supplemented with 6-benzyladenine (BA) at concentrations 0.0–3.2 mg L^{-1} in combination with 0.0, 0.01 and 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA). Infections were the biggest problem for establishing cultures from greenhouse plants, while explants from in vitro seedlings showed hyperhydricity, especially the nodal ones. In the multiplication stage, high BA concentrations reduced explant response to produce shoots as well as the length of shoots produced, and although they increased shoot number per responding explant, a large number of shoots were hyperhydrated especially in explants of seedling origin. In general, the highest multiplication indexes, relevant to normal shoots, were given by the hormone-free medium or one with low BA concentration, and by shoot tip explants originated from greenhouse adult plants. The highest rooting rates (70–78%) were achieved either at full or half strength hormone-free or with 0.1 mg L^{-1} indole-3-butyric acid (IBA) medium. Increasing IBA resulted in an increase in root number, but a decrease in rooting rate and root length. Ex vitro acclimatization was highly successful (92–96%) in peat-perlite (1:1 *v*/*v*) substrate. The micropropagation protocol developed will contribute to the promotion of *S. tomentosa* in the floriculture and pharmaceutical industry and will be a tool for breeding programs targeting high value products.

Keywords: auxin; balsamic sage; benzyladenine; hyperhydricity; micropropagation; shoot multiplication; acclimatization

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1. Introduction

Salvia tomentosa Miller (*S. grandiflora* Etling, Lamiaceae), balsamic sage or tomentosa sage, is a strongly aromatic, medicinal, perennial semi-woody herbaceous plant, up to 80 cm, which has grey-green leaves with a rounded or heart-shaped base and flowers usually violet with reddish-brown calyces in late spring or early summer [1,2]. It grows in areas of maquis vegetation and on limestone slopes, while its geographical distribution extends from South Eastern Europe to Transcaucasia, as well as Crimea, Turkey, Lebanon and Syria [3]. In Greece, it spreads in the northeast and in the northeastern and eastern Aegean Islands [4]. In traditional medicine, *S. tomentosa* is used to heal wounds [5] and relieve stomach and abdominal pain [6], while it is consumed as an herbal tea in some Mediter-

ranean countries [7]. The aerial parts of the plant have antimicrobial and antioxidant properties, due to the significant quantities in secondary metabolites, such as phenolics and terpenoids [8–10]. Essential oil composition of *S. tomentosa*, along with other Mediterranean *Salvia* species, has also been investigated [11–14], and the essential oil diversity in Greek *S. tomentosa* clones has been pointed out [15]. Due to its medicinal properties, *S. tomentosa* is widely traded in Turkey, which may lead to the risk of it becoming an endangered species, because of over-collection from natural populations [16].

The botanical characteristics of *Salvia tomentosa*, its adaptation to xerothermic conditions and at the same time its adaptability to wet conditions and resistance to low temperatures make it suitable for wide ornamental use, in addition to its medicinal and culinary use. The species has been tested under greenhouse conditions concerning its cultivation requirements for ornamental plant production [17] and has been shown appropriate for use on extensive green roofs in the eastern Mediterranean region [18]. It has also been proved capable of being cultivated in low and medium saline soils and of being irrigated with second-class waters [19]. It has also been efficiently cultivated regarding herbage yield, as well as essential oil and phenolic content [7,12], while its essential oil yield could be increased under salt stress [19]. All the above characteristics of its cultivation indicate that it could be used more widely in degraded soils of semi-arid and arid regions from 0 to 2000 altitude.

In the context of a research program (SALVIA-BREED-GR) with the aim of exploiting Greek sage species as ornamentals and their use in interspecific crosses for the production of new highly marketable products in the ornamental sector, the propagation [20,21] and growth [17] of Greek *S. tomentosa* clones were investigated. *S. tomentosa* seeds germinated in vitro at extremely low percentage (<7%), even after mechanical and chemical scarification [20], verifying that the main global problem of *Salvia* spp. is the germination of their seeds [22,23]. In nature *Salvia* seeds germinate very slowly, after having remained dormant for a long time [24]. A higher, but still not satisfactory, seed germination percentage (~40%) of *S. tomentosa* has been reported when cold storage followed by immersion in gibberellin solution was applied [25]. Clonal propagation by spring stem cuttings was very satisfactory [21]. Rooting of cuttings is the method employed for commercial propagation of sage species; however, its efficiency may depend on season or climatic conditions.

Due to the difficulties in seed germination of *S. tomentosa* and its multiple uses as an aromatic, medicinal, ornamental and landscape plant, its propagation by tissue culture should also be investigated. Micropropagation technique is important to select, multiply and conserve the critical genotypes of medicinal and aromatic plants (MAPs) [26]. At the same time, it has the benefits of the large-scale multiplication of disease-free plants, faster cloning and the conservation of desired genotypes in a very short time, along with the potential for the production of active compounds and standardized quality phytopharmaceutical for herbal and pharmaceutical industries [27–31]. Micropropagation is a tool to conserve valuable native plant species and to massively produce high-value plant material for cultivation without seasonal constraints [26]. Micropropagation protocols are worked out for many native plant species cultured in vitro to obtain high regeneration rates, aiming to facilitate commercially feasible micropropagation and enable their possible sustainable use [26,30–33].

To the best of our knowledge, no studies have been performed on the in vitro propagation of *S. tomentosa*. Only callus has been induced from seeds with the aim of using it in secondary metabolite production [34]. However, there are many medicinal Lamiaceae plants, which have been successfully propagated by tissue culture [35]. There are studies concerning micropropagation of the most commercial *Salvia* species, i.e., *S. officinalis*, in which 6-benzyladenine (BA) at concentration 0.5 or 1.0 mg L^{-1}, alone or in combination with 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA), was tested for shoot proliferation [36–38], being found superior to kinetin [36], thidiazuron, zeatin and 2iP [37]. An even lower

BA concentration (0.2–0.5 mg L^{-1}) was found appropriate for shoot proliferation of *S. fruticosa* [39]. On the other hand, higher BA concentrations (1.0–8.0 mg L^{-1}), with or without low auxin concentration, were required for optimal shoot proliferation of *Salvia splendens* [40] and other medicinal Lamiaceae herbs [41–47].

Both the nature and the origin of explants may often play a major role in their in vitro development on a certain type of medium and under specific environmental conditions [48]. Tissue culture success mainly depends on the age, type and position of explants [49–52], because not all plant cells have the same ability to express totipotency.

Explant type is a factor that can affect shoot proliferation in a number of species of the genus *Salvia*, as well as in other medicinal Lamiaceae herbs, where nodal explants have been found to produce more shoots than shoot tip explants [39–45,53,54]. However, there are medicinal Lamiaceae herbs where shoot tips were found superior to nodal explants in shoot proliferation [46], while in varieties of *Lavandula* sp. higher values were obtained from stem node explants in terms of shoot number and from shoot tip explants in terms of shoot length [55].

The use of seedlings as mother plants for micropropagation could lead to a high proliferation rate as has been shown for other native Mediterranean species [41,56]. Furthermore, the use of seedlings for either in vitro or ex vitro propagation could encourage the higher genetic diversity that is desirable when native plants are reintroduced in the landscape or contribute to the selection of specific genotypes of high medicinal value.

Rooting of medicinal Lamiaceae, including species of *Salvia* sp., presents no difficulties [38–47,53–55,57]. In most of the cases referred above, half strength MS medium was recommended for rooting, with a few exceptions where full-MS medium was used [43,54]. IBA, more often at concentrations 0.5–1.5 mg L^{-1} but up to 4.0 mg L^{-1}, was used in the aforementioned studies for in vitro rooting of medicinal Lamiaceae species.

In this work, the micropropagation of *S. tomentosa* was studied for the first time and is one of the steps toward achieving the goal of promoting the species for ornamental and medical use, as well as for breeding purposes. Due to the difficulties in *S. tomentosa* seed germination, micropropagation could lead to mass production of high-value plant material for cultivation without seasonal limitations and the selection, propagation and conservation of critical genotypes. The effect of (a) explant origin, i.e., greenhouse adult plants and seedlings grown in vitro, (b) explant type, i.e., shoot tip and nodal, and (c) type and concentration of plant growth regulators of the culture medium, in all stages of in vitro culture were investigated. The aim was to develop an efficient micropropagation protocol that could facilitate and promote the wider commercial exploitation of the native sage of northeastern Greece *S. tomentosa*.

2. Materials and Methods

2.1. In Vitro Culture Establishment Stage

Four types of in vitro cultures were established, i.e., cultures initiated from (a) shoot tip explants excised from greenhouse adult plants, (b) single-node stem explants excised from greenhouse adult plants, (c) shoot tip explants excised from seedlings grown in vitro and (d) single-node stem explants excised from seedlings grown in vitro.

2.1.1. Explants from Greenhouse Adult Plants (Adult Origin Explants)

Explants of adult origin were excised from stock plants grown in the greenhouse, in July and September 2019 and April 2020. The stock plants were produced by cuttings collected from native, mature *S. tomentosa* plants [21], and were three-months old in July 2019. Four days before establishment of the initial cultures, plants were sprayed with a combination of two systemic fungicides, i.e., fosetyl-Al 80% *w*/*w* (Aliette 80 WG, Bayer, Hellas) at concentration 5 g L^{-1} and thiophanate methyl 70% *w*/*w* (Neotopsin 70 WG, K&N Efthymiadis, Greece) at 1 g L^{-1}. Explants were then excised from shoots 10–15 cm long collected from stock plants, which did not flower during the first year of their culture.

The shoot tip and single-node stem explants of the 1st, 2nd, 3rd and 4th visible node below the shoot tip were used as explants. The explants were about 1.0 cm long.

Before explant excision, the stock shoots were thoroughly rinsed with plenty of tap water followed by surface sterilization in an aqueous solution of 25% commercial bleach (4.6% *w*/*v* NaClO), which contained 0.1% Tween 20 (emulsifying agent, Polyoxyethylene (20) sorbitan Monolaurate) under constant stirring, for 10 min. Surface sterilization was followed by four 3-min rinses with sterile distilled water, under constant stirring.

Planting of explants was done on solid (8 g L^{-1} agar) MS medium [58] with 30 g L^{-1} sucrose without plant growth regulators (Hf).

2.1.2. Explants from Seedlings Grown In Vitro (Juvenile Origin Explants)

Explants of juvenile origin were excised from 3-month-old in vitro grown seedlings in March 2019. The seedlings were germinated in vitro, following the method described by Vlachou et al. [20], and grown on Hf-MS medium with 30 g L^{-1} sucrose, under culture conditions described below.

The shoot tip and single-node stem explants of the 1st, 2nd and 3rd visible node below the shoot tip were used as explants. The explants were about 0.6 cm long.

Planting of explants was done on solid (8 g L^{-1} agar) Hf-MS medium with 30 g L^{-1} sucrose.

2.2. Shoot Multiplication Stage

2.2.1. Subcultures to Obtain Sufficient Number of Microshoots/Effect of BA/NAA

Six subcultures were performed in order to obtain sufficient plant material (microshoots) for further experimentation on shoot proliferation. In these subcultures were preserved the origin of the initial cultures (greenhouse adult plants or in vitro seedlings), but not the explant type (shoot tip or nodal) from which the initial cultures were established. Therefore, we had two types of cultures, i.e., one originating from greenhouse adult plants and a second originating from in vitro seedlings.

In these six subcultures, shoot tip and single node explants, 0.6 cm long, were cultured on MS medium with 30 g L^{-1} sucrose, either Hf (control) or supplemented with 0.4 mg L^{-1} BA and 0.01 mg L^{-1} NAA. Thus, apart from multiplying the in vitro material for further experimentation, the effect of BA/NAA presence on shoot proliferation was examined. Although data were recorded separately for shoot tip and single node explants response, in the results section data are pooled because there were no statistical significant differences concerning the effect of explant type on normal (not hyperhydrated) shoot production.

2.2.2. Effect of BA Concentration and Explant Type and Origin on Proliferation

The aim of this experiment was to investigate the effect of BA concentration, in combination with explant type (shoot tip, single node) and origin (initial cultures established from either greenhouse adult plants or in vitro seedlings), on shoot proliferation. Shoot tip and single node explants of both origins were cultured either on the Hf-MS medium or on MS medium supplemented with four BA concentrations, 0.4, 0.8, 1.6 and 3.2 mg L^{-1}, in combination with 0.01 mg L^{-1} NAA.

The explants were 0.6 cm long and were excised from microshoots grown in the 6th subculture on MS medium supplemented with 0.4 mg L^{-1} BA and 0.01 mg L^{-1} NAA. Data are presented separately for shoot tip and single node explants and for explant origin (greenhouse adult plants and in vitro seedlings).

2.2.3. Effect of BA and NAA Concentration and Explant Type on Proliferation

The aim of this experiment was to investigate the effect of NAA concentration on shoot proliferation simultaneously with that on hyperhydricity. Shoot tip and single node

explants, 0.6 cm long, were excised from microshoots of cultures originated from seedlings and produced on MS medium supplemented with 0.4 mg L^{-1} BA and 0.01 mg L^{-1} NAA. The explants were cultured either on the Hf-MS medium or on MS medium supplemented with 0.4, 0.8, 1.6 or 3.2 mg·L^{-1} BA in combination with 0.0, 0.01 or 0.1 mg L^{-1} NAA.

2.2.4. Explant Number, Data Collection at shoot multiplication stage

Number of explants used in each experiment at the multiplication stage is presented separately at the base of each the relevant data Table.

Data were collected after 30 d of culture. The "multiplication index" of each culture was calculated by multiplying the percentage of explants that produced normal shoots (not hyperhydrated) by the mean number of normal shoots per responding explant, and by the mean node number per normal shoot.

2.3. In Vitro Rooting

For the experiment on in vitro root induction and development, microshoots of both origins (greenhouse adult plants and in vitro seedlings), produced on MS medium supplemented with 0.4 mg L^{-1} BA and 0.01 mg L^{-1} NAA were used, when about 2.5 cm long. The microshoots were cultured on full- or half- strength MS medium, symbolized as MS or ½MS, respectively, with 20 g L^{-1} sucrose and supplemented with various IBA concentrations, i.e., 0.0, 0.1, 0.25, 0.5, 1.0, 2.0 or 4.0 mg L^{-1}.

Five replicates of 10 microshoots were used for each treatment. Data were collected after 30 d of culture.

2.4. Ex Vitro Acclimatization

For acclimatization, rooted microshoots of both origins (greenhouse adult plants and in vitro seedlings) and all IBA concentrations of the rooting medium were transferred ex vitro into trays (eight plantlets per 500-mL volume tray) with a mixture of peat: perlite (1:1, *v*/*v*). The trays were covered with plastic wrap (SANITAS; Sarantis S.A., Athens, Greece) and placed in a growth chamber (20 °C and 16-h cool white fluorescent light 37.5 mmol·m^{-2}·s^{-1}) for 1 week before their transfer to a heated greenhouse.

Ten replicates of eight rooted microshoots were used and their survival was estimated at 30 d after transfer to the greenhouse.

2.5. In Vitro Culture Conditions

All media were solidified with 8 g L^{-1} agar and their pH was adjusted to 5.7 before agar addition and autoclaving (121 °C for 20 min). Initial cultures from greenhouse plants took place in test tubes (25 × 100 mm) with 10 mL medium (one explant per tube), covered with plastic wrap, while initial cultures from in vitro seedlings, subcultures and rooting experiments took place in 145-mL glass vessels with 25 mL medium (four explants or microshoots per vessel), covered with magenta plastic cap. The cultures were maintained at 25 °C with a 16 h photoperiod at 37.5 μmol m^{-2} s^{-1} fluorescent light, provided by cool-white fluorescent lamps.

2.6. Statistical Analysis

A completely randomized design was used. The significance of the results was tested by either one- or two- or three-way analysis of variance (ANOVA) and the means of the treatments were compared by Student's *t* test at $p \leq 0.05$ (JMP 13.0 software, SAS Institute Inc., Cary, NC, USA).

3. Results and Discussion

The floriculture industry internationally aims to continuously introduce new species to the market in order to keep the purchasing interest of the public high. Native species

are an important reservoir for the development of new floricultural products. Following the current tendency for xeriscaping in landscaping and gardening, and taking into consideration the various ecological, environmental, economic and aesthetic advantages of using native plants, *Salvia* species, part of the maquis vegetation, could be ideal for exploitation as ornamental plants. Being drought resistant species could be valuable for introduction as landscape plants particularly in arid and semi-arid regions, where water should be used very sparingly for irrigation purposes.

At the same time, most *Salvia* species are valuable as medicinal plants. Medicinal plants are still the major source of therapies for several illnesses, having important an role in health care systems [28,59]. The production and consumption of medicinal plants and herbal medicines, as well as the international trade of medicinal plants and phytomedicines, are growing quite significantly. However, the harvesting of herbal medicine from the wild represents the major supply of raw material, which may cause loss of genetic diversity and habitat destruction [10,31], and which threatens the quality and safety of the final products [59]. This is why it is important to develop medicinal plants into crops, and the use of micropropagation as a technique to mass produce high-demand biomass could solve the supply issues of therapeutic natural substances [10,59], ensuring their required qualitative and quantitative uniformity as well [60]. At the same time, micropropagation supplies the floriculture industry with high-quality propagation material and is a tool for breeding programs.

S. tomentosa is a plant used in traditional medicine [5,6], rich in antioxidant and antimicrobial substances and essential oils [7–15], with particular botanical characteristics and adaptation to xerothermic conditions of the eastern Mediterranean region [18,19], which make it useful for both pharmaceutical and floriculture industry. This is the first time that a micropropagation protocol for *S. tomentosa* has been developed. Micropropagation will provide an alternative to propagation by seed, which exhibits poor germination ability, [20,25] and to propagation by cuttings, the efficiency of which may depend on seasonal and climatic conditions. This will allow its wider exploitation as an ornamental plant, while it will promote its development into a crop for medicinal uses that will stop its overharvesting from natural populations, thus protecting biodiversity.

3.1. Establishment of In Vitro Cultures

3.1.1. From Greenhouse Adult Plants

When explants were excised from adult plants grown in a greenhouse, the infection rate of the explants at the in vitro establishment stage was very high, ranging between 25 and 65% depending on the type of explant and the season of establishment attempt (Table 1). Lower nodes showed the highest rates of infection, while shoot tips were burned by the bleach solution at the higher rates (63% in April, 42% in July) compared to nodal explants (30% in April, 12% in September). From the few explants that survived in each trial, those of the 1st and 2nd nodes were the only ones that responded by producing shoots. The highest response was found in the culture established in April, while in September none of the explants produced shoots (Table 1, Figure 1a).

Initiation of in vitro cultures from adult wild plants may be even more difficult or unsuccessful [30]. If plantlets of selected adult genotypes are produced by cuttings and remain for growth inside a greenhouse to be used as explant source for in vitro culture initiation as an intermediate stage, then micropropagation of selected genotypes is feasible with less difficulties [41]. This can possibly cause "rejuvenation" of explant tissues [61,62] and reduce contamination risk. So, the successful establishment of *S. tomentosa* from greenhouse plants is important as method for in vitro propagation of selected wild plants, following the intermediate stage described previously.

Table 1. Effect of season and origin and type of explant on axillary shoot production of *S. tomentosa* at the in vitro culture establishment stage on a hormone-free MS medium.

Explant Origin	Season	Explant Type	Explant Infection (%), (n = 24)	Explant Burning (%)	Shoot-Producing Explants [1/2] (%)	Mean Number of NSh [Ŧ]/ HSh [ŦŦ]	Mean NSh Length (cm)	Mean NSh Node Number	Multi Plication Index [Ŧ]
Greenhouse adult plants	19 July	Shoot tip	33	42	0	–/–	–	–	–
		1st node	25	17	21 b [z]/ 0 c	1.3 b/–	2.5 a	2.7	0.7 b
	19 September	Shoot tip	29	33	0	–/–	–	–	–
		1st node	38	20	0	–/–	–	–	–
		2nd node	25	21	0	–/–	–	–	–
		3rd node	65	12	0	–/–	–	–	–
	20 April	Shoot tip	37	63	0	–/–	–	–	–
		1st, 2nd node	58	30	71 a/0 c	1.8 a/0 b	0.8 b	2.0	2.6 a
		3rd, 4th node	54	33	0	–/–	–	–	–
Seedlings in vitro	19 March	Shoot tip	0	0	26 b/10 b	1.0 b/0.2 b	1.9 b	2.4	0.6 b
		1st, 2nd, 3rd node	0	0	10 c/40 a	2.0 a/0.9 a	1.4 c	2.0	0.6 b
$F_{\text{one-way ANOVA}}$					***/***	**/***	***	NS	***

[z] Mean separation in columns by Student's t, $p \leq 0.05$; means followed by the same letter are not significantly different at $p \leq 0.05$. NS: nonsignificant or **, *** significant at $p \leq 0.01$, $p \leq 0.001$, respectively, n = 7–14. Multiplication index = shoot-producing explants [1] (%) × mean shoot number [Ŧ] × mean node number [Ŧ]. [1] The explants produced normal and hyperhydrated shoots. [2] The explants produced hyperhydrated shoots only. [Ŧ] NSh = normal shoot. [ŦŦ] HSh = hyperhydrated shoot.

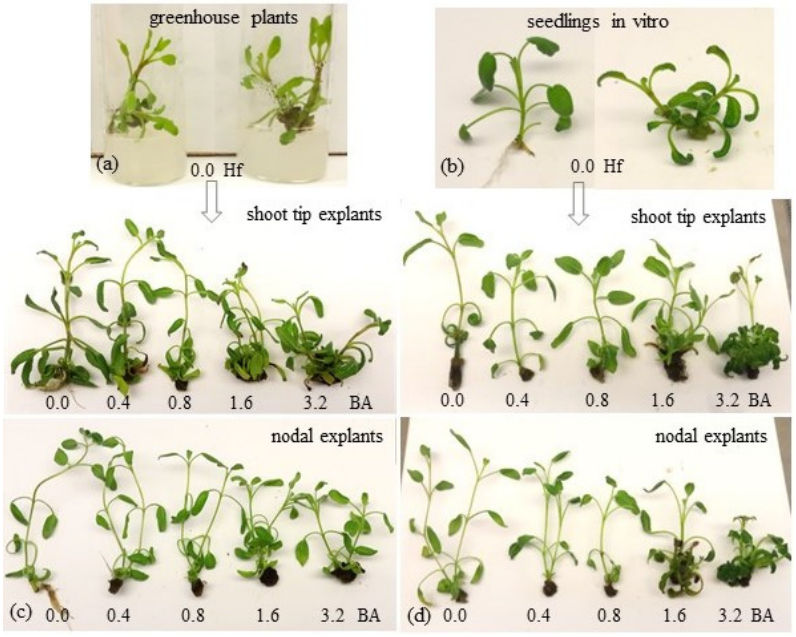

Figure 1. Typical response of *S. tomentosa* shoot tip (left) and single-node (right) explants excised from greenhouse adult plants (**a**) and in vitro seedlings (**b**), at the in vitro establishment stage in a hormone-free (Hf) MS medium; axillary shoot proliferation of shoot tip and single-node explants of greenhouse adult plants (**c**) and in vitro seedlings (**d**) origin, at a subculture on MS medium without plant growth regulators (0.0) or supplemented with 0.4 to 3.2 mg L^{-1} BA and 0.01 mg L^{-1} NAA.

3.1.2. From In Vitro Seedlings

When explants were excised from seedlings grown in vitro, 26% of the shoot tip explants responded by forming one normal shoot, while 10% of the explants showed hyperhydricity. Hyperhydricity was much higher in the 1st and 2nd nodes, where only 10% of explants produced normal shoots and 40% produced only hyperhydrated shoots (Table 1). Thus, although two shoots per explant sprouted, the multiplication index was as low as in shoot tip explants and only the explant origin had a significant effect on the length of normal shoots as having a greenhouse origin resulted in longer microshoots (Table 1, Figure 1b). Regarding the relative species *S. officinalis*, in which corresponding explants were used, both explant types established in vitro cultures at high percentage (75–93%, higher the shoot tip ones), whereas many explants, especially the nodal ones, formed hyperhydrated shoots [63], as happened in the present study, too.

Culture initiation is arguably the most critical stage of micropropagation because the potential for multiple shoot generation lies within single buds and predetermined growth from apical and axillary buds could still be expressed in culture [64]. Propagation from pre-existing meristems by shoot tip and node culture is the most reliable and widely used method for micropropagation of many species including MAPs [30]. However, in the in vitro establishment of *S. tomentosa* the percentage of necrosed explants that did not respond was high for both explant origins, while a high percentage of hyperhydrated shoots was presented in case of explants from in vitro seedlings. These difficulties were also reported for culture initiation of *S. officinalis* from either two-year old plants [36] or in vitro seedlings [63].

Although explants excised from in vitro seedlings usually establish in vitro culture more efficiently than other adult explant sources from greenhouse or wild plants—something which has been verified in the case of other Lamiaceae, such as *S. officinalis* [63] and *Teucrium capitatum* [41], and in other shrubs and trees, particularly in difficult to propagate woody species [52,56,65–70]—in the present study *S. tomentosa* explants derived from in vitro seedlings showed a similarly poor establishment ability as explants derived from greenhouse adult plants. The use of seedlings as stock material for micropropagation of native plants is indicated when the plants are reintroduced to the landscape or when the goal is to select genotypes of high medicinal value, as it could encourage the higher genetic diversity that, in these cases, is desirable.

3.2. Shoot Multiplication Stage

3.2.1. Subcultures to Obtain Sufficient Number of Microshoots/Effect of BA/NAA

The explant response was consistent between subcultures that took place on Hf-MS medium and MS medium supplemented with 0.4 mg L^{-1} BA and 0.01 mg L^{-1}, so the data of six subcultures were pooled (Table 2). Almost all explants produced normal shoots regardless of explant origin, with the exception of seedling origin explants cultured on medium with 0.4 mg L^{-1} BA and 0.01 mg L^{-1}, which showed relatively high hyperhydricity, as 23% of the explants produced only hyperhydrated shoots (Table 2). The multiplication index was only slightly increased compared with the establishment stage, being higher in cultures that originated from greenhouse adult plants due to the longer shoots they produced and the increased nodes present on these shoots as compared with the seedling-origin cultures (Table 2).

Table 2. Effect of explant origin and plant growth regulators in the medium on axillary shoot proliferation of *S. tomentosa* shoot tip and single node explants (mixed) at subcultures (means of six subcultures).

Explant Origin	BA/ NAA (mg L^{-1})	Shoot-Producing Explants [1/2] (%)	Mean Number of NSh [Ŧ]/ HSh [ŦŦ]	Mean NSh Length [Ŧ] (cm)	Mean NSh Node Number [Ŧ]	Multiplication Index [Ŧ]
Green house adult plants	0.0/0.0	92 a [z]/6 c	1.4 b/0.3 b	2.1 b	2.5 ab	3.1 ab
	0.4/0.01	90 a/9 b	1.4 b/0.3 b	2.7 a	2.9 a	3.8 a
Seedlings in vitro	0.0/0.0	92 a/8 b	1.1 b/0.2 b	1.4 b	2.1 b	2.1 b
	0.4/0.01	73 b/23 a	1.5 a/0.7 a	1.5 b	2.0 b	2.1 b
$F_{BA/NAA\ concentration}$		−/−	NS/−	NS	NS	NS
$F_{explant\ origin}$		−/−	NS/−	***	**	***
$F_{BA/NAA\ conc.\ \times\ explant\ origin}$		***/***	NS/***	NS	NS	NS
$F_{one\text{-}way\ ANOVA}$		***	*/***	***	***	***

[z] Mean separation in columns by Student's *t*, $p \leq 0.05$; means followed by the same letter are not significantly different at $p \leq 0.05$. NS: nonsignificant or *, **, *** significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively, n = 10–85. Multiplication index = shoot-producing explants [1] (%) × mean shoot number [Ŧ] × mean node number [Ŧ]. [1] The explants produced normal and hyperhydrated shoots. [2] The explants produced hyperhydrated shoots only. [Ŧ] NSh = normal shoot. [ŦŦ] HSh = hyperhydrated shoot.

3.2.2. Effect of BA Concentration and Explant Type and Origin on Proliferation

When, in a subsequent experiment, the effect of BA concentration in the medium was tested in relation to the origin and type of explant, there was a significant interaction of the experimental factors (Table 3). However, there was an indication that the increase in BA concentration resulted in a decrease in the explant response in terms of the production of normal shoots and an increase in the rate of hyperhydricity (Table 3, Figure 1c,d). The highest concentration of BA tested (3.2 mg L^{-1}) led to the highest inhibition of explant response mainly reducing the production of normal shoots. A similar response was shown in *Mentha piperita*, where hyperhydrated shoots were formed and the production of shoots was reduced at BA concentrations higher than 2.0 mg L^{-1}, indicating an inhibitory effect of cytokinin at high concentrations on proliferation [42]. Hyperhydricity syndrome is expressed through waterlogging of the apoplast, which is caused by the abundant availability of water in the medium and the high humidity inside the tissue culture containers, which reduces transpiration from the leaves [71]. Stresses imposed during tissue culture, including gelling agent, wounding, exposure to high levels of plant growth regulators and ammonium ions and a strongly modified atmosphere are reasons that induce hyperhydricity [72,73], while natural ventilation of the cultures [74], increase of the gelling agent concentration and a balance between cytokinin and auxin in the medium can control hyperhydricity [46,47].

At the same time, the highest BA concentration resulted in the highest shoot production per responding explant in total, normal and hyperhydrated shoots pooled, but at least half of the shoots were hyperhydrated and in explants of seedling origin the rate was reversed and hyperhydrated shoots were twice as many as normal shoots (Table 3). In previous studies on the micropropagation of various *Salvia* species, such as *S. fruticosa* [39], *S. officinalis* [38,63], *S. sclarea* [75] and *S. wagneriana* [76], respectively, low BA concentration (0.2–0.5 mg L^{-1}) favored shoot proliferation, in most cases combined with low auxin concentration, whereas higher BA concentrations (1.0–3.0 mg L^{-1}) were required for optimal shoot proliferation of *Salvia splendens* [40] and *Salvia hispanica* [57], as well as other medicinal Lamiaceae [41,43–45].

Generally, for explants that responded by producing normal shoots, increasing BA resulted in a slight increase in shoot production per explant, mainly in shoot tip explants,

while decreasing shoot length (Table 3, Figure 1). Explants originated from greenhouse adult plants produced the highest number of normal shoots per explant (Table 3). Shoot tip explants responded at higher percentages to form shoots without hyperhydricity compared with nodal ones, especially at the lower BA concentrations, which has also been reported for *S. officinalis* [63].

Thus, in general, the highest multiplication indexes, relevant to normal shoots, were given by the Hf medium or one with low BA concentration, and by shoot tip explants of greenhouse adult plant origin (Table 3). Explants, regardless of type and origin, rooted when cultured on Hf medium (Figure 1). Explant type did not have such a significant effect on *S. tomentosa* shoot proliferation, which was mainly affected by BA concentration. In contrast, in other *Salvia* species and medicinal Lamiaceae herbs, maximum shoot proliferation has been achieved by nodal explants compared to shoot tips [39–40,42–45,53,54].

Table 3. Effect of origin and type of explant and BA concentration in the medium on axillary shoot proliferation of *S. tomentosa* at a subculture in the presence of 0.01 mg L^{-1} NAA. Explants were excised from microshoots grown on MS medium with 0.4 mg L^{-1} BA and 0.01 mg L^{-1} NAA.

Explant Origin	BA (mg L^{-1})	Shoot-Producing Explants [1/2] (%)	Mean Number of NSh [Ŧ]/ HSh [ŦŦ]	Mean NSh Length [Ŧ] (cm)	Mean NSh Node Number [Ŧ]	Multiplication Index [Ŧ]
Greenhouse adult plants	Shoot tip explant					
	0.0 (Hf [ŦŦŦ])	96 a [z]/4 l	1.0 i/0.1 k	6.4 bc	5.7 b–d	5.5 ab
	0.4	92 a/8 k	1.0 i/0.1/k	7.2 b	6.5 b	6.0 a
	0.8	7 b/13 j	1.1 hi/0.1 k	6.1 cd	5.9 bc	5.6 ab
	1.6	61 e/29 c	2.2 b/0.7 g	4.3 f–i	4.8 ef	6.4 a
	3.2	46 f/33 b	2.6 a/1.5 d	3.2 h–k	3.4 gh	4.1 cd
	Single node explant					
	0.0 (Hf [ŦŦŦ])	63 de/17 i	1.2 hi/0.3 j	8.7 a	7.6 a	5.7 ab
	0.4	50 f/21 h	1.9 b–d/0.4 j	5.5 c–f	5.0 c–e	4.8 bc
	0.8	38 g/33 b	2.0 bc/0.9 f	3.7 g–k	4.1 e–g	3.1 d–f
	1.6	29 h/40 a	2.0 b–d/1.2 e	3.2 h–k	3.6 f–h	2.1 f–i
	3.2	17 j/38 a	2.0 b–d/1.5 e	2.8 h-k	4.3 e–g	1.5 f–i
Seedlings in vitro	Shoot tip explant					
	0.0 (Hf [ŦŦŦ])	69 c/28 c–e	1.0 i/0.3 j	5.1 d–g	4.8 ef	3.3 de
	0.4	64 de/28 cd	1.0 i/0.4 j	4.6 e–h	3.8 fg	2.4 e–g
	0.8	66 cd/27 d–f	1.3 e–h/0.3 j	3.4 h–k	3.4 gh	2.9 ef
	1.6	59 e/29 c	1.7 c–f/0.5 j	2.9 h–k	2.6 h	2.6 e–g
	3.2	21 ij/25 fg	1.3 e–h/2.6 b	3.1 h–k	3.1 gh	0.8 i
	Single node explant					
	0.0 (Hf [ŦŦŦ])	67 cd/25 g	1.6 d–g/0.6 hi	5.8 c–e	5.0 c–e	5.4 ab
	0.4	48 f/26 e–g	1.6 c–g/0.7 gh	4.2 f–j	3.4 gh	2.6 e–g
	0.8	27 hi/33 b	1.7 c–f/1.1 e	2.3 jk	2.3 h	1.1 hi
	1.6	29 h/17 i	1.3 e–h/2.1 c	3.1 h–k	3.4 gh	1.3 g–i
	3.2	16 j/13 j	1.3 e–h/2.9 a	2.3 k	2.8 gh	0.6 i
F_{BA}		–/–	–/–	–	–/–	–
$F_{expl.\ origin}$		–/–	–/–	***	–/–	–
$F_{expl.\ type}$		–/–	–/–	–	–/–	–
$F_{BA \times expl.\ type}$		–/–	–/–	***	–/–	***
$F_{BA \times expl.\ origin}$		–/–	–/–	NS	–/–	*
$F_{expl.\ origin \times expl.\ type}$		–/–	–/–	NS	–/–	***
$F_{BA \times origin \times type}$		***/**	*/***	NS	***	NS
$F_{one\text{-}way\ ANOVA}$		***/***	***/***	***	***	***

[z] Mean separation in columns by Student's *t*, $p \leq 0.05$; means followed by the same letter are not significantly different at $p \leq 0.05$. NS: nonsignificant, or *, **, *** significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively, $n = 30$. Multiplication index = shoot-producing explants [1] (%) × mean shoot number [Ŧ] × mean node number [Ŧ]. [1] The explants produced normal and hyperhydrated shoots. [2] The explants produced hyperhydrated shoots only. [Ŧ] NSh = normal shoot. [ŦŦ] HSh = hyperhydrated shoot. [ŦŦŦ] Hf = hormone free.

3.2.3. Effect of BA and NAA Concentration and Explant Type on Proliferation

Aiming to increase shoot proliferation and reduce hyperhydricity, in a subsequent experiment the medium was supplemented either with BA alone at the concentrations previously tested, or with BA at these concentrations combined with NAA at the low concentration previously used or at a tenfold concentration. In this experiment, there was also a significant interaction of the experimental factors in most parameters recorded, apart from the number of normal shoots produced per explant, which was slightly promoted by the higher NAA concentration, and the mean length and node number of normal shoots, which was slightly reduced by the increase of BA concentration (Table 4). However, neither the addition of NAA in the medium nor the increase of NAA concentration resulted in a decrease of hyperhydricity, in contrast to previous works with other Mediterranean species, *Clinopodium nepeta* [46], *Calamintha cretica* [47] and *Anthyllis barba-jovis* [77].

Table 4. Effect of plant-growth regulator type and concentration and explant type on axillary shoot proliferation of *S. tomentosa* explants excised from cultures originated from in vitro seedlings. Explants were excised from microshoots grown on MS medium with 0.4 mg L^{-1} BA and 0.01 mg L^{-1} NAA.

BA/ NAA Concentration (mg L^{-1})	Shoot-Producing Explants [1/2] (%)	Mean Number of NSh [Ŧ]/HSh [ŦŦ]	Mean NSh Length [Ŧ] (cm)	Mean Node Number [Ŧ]	Multiplication Index
Shoot tip explant					
0.0/0.0 (Hf [ŦŦŦ])	95 a [z]/5 h	1.0 e/0.1 k	4.2 ab	4.0 ab	3.8 b–d
0.0/0.01	75 cd/15 fg	1.0 e/0.2 j	4.1 abc	3.9 abc	2.9 e–g
0.0/0.1	80 bc/10g	1.0 e/0.1 k	3.7 bcde	3.7 bc	3.0 e–g
0.4/0.0	75 cd/20 de	1.1 de/0.2 j	3.0 cde	3.2 de	2.6 g–i
0.4/0.01	80 bc/20 de	1.1 de/0.2 j	4.5 a	4.0 ab	3.5 c–e
0.4/0.1	85 b/10 g	1.3 c–e/0.1 k	4.2 ab	3.8 abc	4.2 bc
0.8/0.0	65 e–g/27 bc	1.1 de/0.4 hi	3.8 bcd	3.9 abc	2.8 f–h
0.8/0.01	60 f–h/25 c	1.3 c–e/0.3 ij	3.2 cde	3.4 cde	2.7 f–h
0.8/0.1	65 e–g/20 de	1.4 c–e/0.3 ij	3.0 cde	3.2 cde	2.9 e–g
1.6/0.0	50 i/32 a	1.4 c–e/0.8 fg	2.7 de	3.1 de	2.2 i
1.6/0.01	56 hi/30 ab	1.6 a–c/0.6 gh	2.9 de	2.9 de	2.6 g–i
1.6/0.1	57 g–i/27 bc	1.7 a–c/0.5 gh	2.8 de	3.0 de	2.9 e–g
3.2/0.0	25 lm/28 bc	1.4 c–e/1.8 cd	2.6 de	2.8 de	1.0 kl
3.2/0.01	30 kl/25 c	1.4 c–e/1.4 de	2.7 de	3.0 de	1.3 kl
3.2/0.1	30 kl/20 de	1.5 b–d/1.4 de	2.8 de	3.3 cde	1.5 jk
Single node explant					
0.0/0.0 (Hf [ŦŦŦ])	80 bc/15 fg	1.3 c–e/0.4 hi	4.2 ab	4.2 a	4.4 ab
0.0/0.01	80 bc/15 fg	1.7 a–c/0.4 hi	3.6 bcde	3.8 abc	5.2 a
0.0/0.1	75 cd/17 ef	1.8 ab/0.4 hi	3.5 bcde	3.8 abc	5.1 a
0.4/0.0	60 f–h/30 ab	1.5 b–d/0.6 gh	3.3 cde	3.6 bc	3.2 de
0.4/0.01	65 e–g/20 de	1.6 a–c/0.6 gh	3.0 cde	2.7 de	2.8 f–h
0.4/0.1	70 de/20 de	1.5 b–d/0.5 gh	3.2 cde	2.6 e	2.7 f–h
0.8/0.0	40 j/30 ab	1.6 a–c/1.0 e	3.3 cde	3.7 bc	2.4 hi
0.8/0.01	50 i/30 ab	1.7 a–c/0.8 fg	2.8 de	2.9 de	2.5 g–i

0.8/0.1	54 hi/25 c	1.9 a/0.8 fg	2.7 de	2.9 de	3.0 e–g
1.6/0.0	30 kl/30 ab	1.3 c–e/2.1 b	2.8 de	2.9 de	1.1 kl
1.6/0.01	30 kl/25 c	1.4 c–e/1.8 cd	2.5 e	2.6 e	1.1 kl
1.6/0.1	35 jk/22 e	1.4 c–e/1.9 cd	2.6 de	2.7 de	1.3 kl
3.2/0.0	10 n/20 de	1.2 de/2.2 ab	2.4 e	2.8 de	0.3 m
3.2/0.01	15 mn/17 ef	1.4 c–e/2.5 a	2.6 de	3.2 cde	0.7 lm
3.2/0.1	15 mn/15 fg	1.4 c–e/2.2 ab	2.8 de	3.0 de	0.6 lm
F_{BA}	–/–	–/–	***	***	–
F_{NAA}	–/–	*/–	NS	NS	–
$F_{explant\ type}$	–/–	–/–	*	NS	–
$F_{BA \times NAA}$	–/–	NS/–	NS	NS	–
$F_{BA \times explant\ type}$	–/–	**/–	NS	NS	–
$F_{NAA \times explant\ type}$	–/–	NS/–	NS	NS	–
$F_{BA \times NAA \times expl.\ type}$	*/***	NS/***	NS	NS	***
$F_{one\text{-}way\ ANOVA}$	***/***	***/**	***	*	***

[z] Mean separation in columns by Student's *t*, $p \leq 0.05$; means followed by the same letter are not significantly different at $p \leq 0.05$. NS: nonsignificant, or *, **, *** significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively, $n = 20$. Multiplication index = shoot-producing explants [1] (%) × mean shoot number$^{\overline{\top}}$ × mean node number $^{\overline{\top}}$. [1] The explants produced normal and hyperhydrated shoots. [2] The explants produced hyperhydrated shoots only. $^{\overline{\top}}$ NSh = normal shoot. $^{\overline{\top\top}}$ HSh = hyperhydrated shoot. $^{\overline{\top\top\top}}$ Hf = hormone free.

3.3. In Vitro Rooting

The highest rooting rates (70–78%) were achieved in full- or half-strength Hf medium, as well as the lowest IBA concentration (0.1 mg L^{-1}) tested. Corresponding rooting percentage (75%) was reported for *S. officinalis* [38], while microshoots of many other medicinal Lamiaceae herbs rooted more efficiently at percentages higher than 90% [41–42,44–47,54,57]. In most of the abovementioned studies, MS medium at half strength was proposed for rooting, with a few exceptions where the full-MS medium was used [43,54]. In the present study both half- and full-strength MS medium were similarly efficient for microshoot rooting, when zero or the lowest concentration of IBA was used, which as mentioned, was most appropriate for rooting of *S. tomentosa*. However, when higher IBA concentrations were used, in microshoots originating from greenhouse adult plants, rooting rate, as well as root number and length were promoted by half-strength medium (Table 5, Figure 2a,b), revealing an interaction between nutrients and auxin concentration of the medium on root initiation and growth. An equally low IBA concentration was used in rooting microshoots of *Salvia hispanica* [57], although generally higher IBA concentrations (0.5–1.5 mg L^{-1}, more often, and up to 4.0 mg L^{-1}) were used in the abovementioned studies on in vitro rooting of other medicinal Lamiaceae herbs.

Table 5. Effect of microshoot origin, growth medium and IBA concentration on in vitro rooting of *S. tomentosa* microshoots. The microshoots were produced on MS medium with 0.4 mg L^{-1} BA and 0.01 mg L^{-1} NAA.

Microshoot Origin	Medium	IBA (mg·L^{-1})	Rooting (%)	Mean Root Number	Mean Root Length (cm)
Greenhouse adult plants	MS	0.0	70 b–e [z]	2.0 i	4.6 a
		0.1	70 b–e	2.4 hi	2.8 bc
		0.25	65 e–g	2.4 hi	2.1 d-f
		0.5	60 gh	2.5 g–i	2.0 d–f
		1.0	57 h	3.1 d–i	1.6 e–k
		2.0	40 k	3.7 b–h	1.4 f–k
		4.0	28 l	4.2 b–f	1.2 h–k

		0.0	78 a	3.1 d–h	4.2 a
		0.1	70 b–e	3.2 d–h	4.1 a
		0.25	67 d–f	3.6 c–f	3.1 b
	½ MS	0.5	67 d–f	3.8 b–f	3.1 b
		1.0	67 d–f	4.5 b–d	2.3 cd
		2.0	50 j	4.6 b	2.1 de
		4.0	44 k	6.3 a	1.8 d–h
		0.0	65 e–g	2.8 f–i	1.8 d–h
		0.1	63 fg	3.1 d–i	1.4 g–k
		0.25	65 e–g	3.3 d–h	1.6 e–j
	MS	0.5	57 h	3.4 d–h	1.8 d–i
		1.0	55 hi	4.3 b–d	1.3 h–k
		2.0	30 l	4.5 b–d	1.0 i–k
Seedlings in vitro		4.0	25 l	4.2 b–e	0.8 k
		0.0	75 ab	2.9 f–i	2.2 de
		0.1	75 a–c	3.2 d–h	1.8 d–h
		0.25	70 b–e	3.2 d–h	1.7 d–j
	½ MS	0.5	72 bc	3.4 c–h	1.9 d–g
		1.0	55 hi	3.2 d–h	1.6 e–k
		2.0	50 ij	4.2 b–e	1.2 h–k
		4.0	39 k	4.0 b–f	1.0 i–k
$F_{\text{microshoot origin}}$			–	–	–
F_{medium}			–	–	–
F_{IBA}			–	***	–
$F_{\text{microshoot origin} \times \text{medium}}$			–	***	**
$F_{\text{microshoot origin} \times \text{IBA}}$			–	NS	***
$F_{\text{medium} \times \text{IBA}}$			–	NS	NS
$F_{\text{microshoot origin} \times \text{medium} \times \text{IBA}}$			**	NS	NS
$F_{\text{one-way ANOVA}}$			***	***	***

[z] Mean separation in columns by Student's *t*, $p \leq 0.05$; means followed by the same letter are not significantly different at $p \leq 0.05$. NS: nonsignificant, or **, *** significant at $p \leq 0.01$, $p \leq 0.001$, respectively, $n = 50$.

A gradual increase in IBA resulted in a gradual decrease in rooting rate and simultaneously a gradual increase in root number and a decrease in root length (Table 5, Figure 2). The increase in IBA had the same effect, as in the present study, on the number and length of roots of *Teucrium capitatum* microshoots [41], with the difference that the percentage of rooting also increased, unlike *S. tomentosa*. On the other hand, the presence of IBA in the medium increased root length in *Clinopodium nepeta* regardless of concentration [46]. Microshoot origin had no effect on rooting rate, while there was an indication that greenhouse adult-plant origin and half-strength medium slightly promoted root number and length (Table 5, Figure 2a,b). Neither *S. tomentosa* nor other Lamiaceae herbs mentioned above presented differences in microshoot rooting rate caused by microshoot origin, which could be attributed to the herbaceous nature of their shoots. On the contrary, microshoots of many woody plants from cultures of juvenile origin were more capable of rooting than those from cultures established from adult plants [51–52,78–79]. In other woody species, however, the effect of age of the explant source was not observed at rooting stage [68].

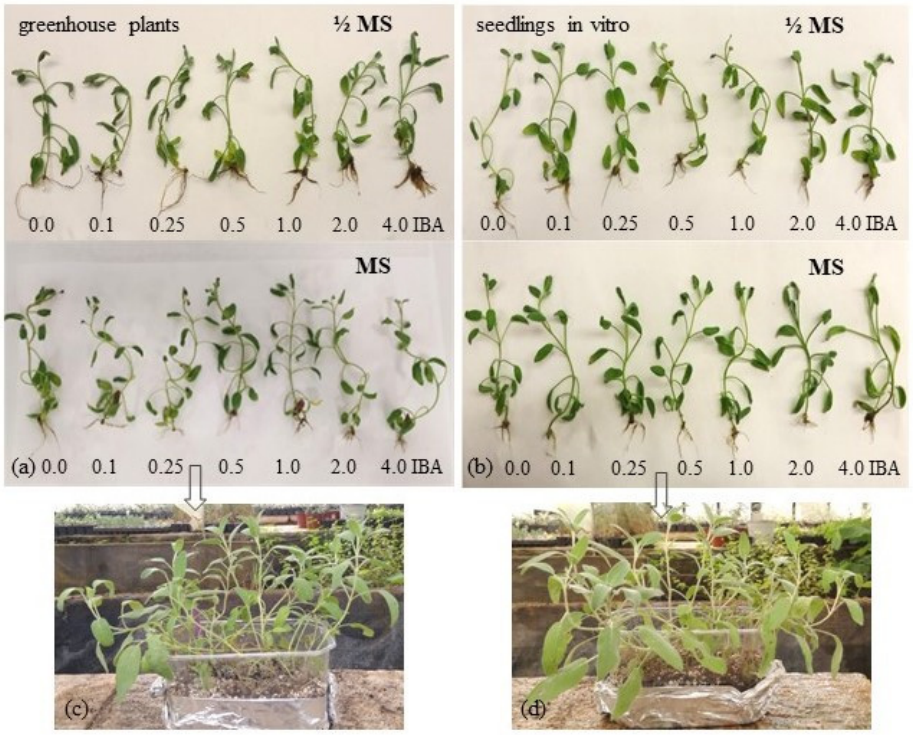

Figure 2. Typical rooting response of *S. tomentosa* microshoots originated from greenhouse adult plants (**a**) and in vitro seedlings (**b**), in either half- or full-strength MS medium supplemented with 0.0 to 4.0 mg L^{-1} IBA; one-month old plantlets originated from either greenhouse adult plants (**c**) or in vitro seedlings (**d**) acclimatized ex vitro.

3.4. Ex Vitro Acclimatization

Plantlets (rooted microshoots) originated from either greenhouse adult plants or in vitro seedlings and transferred ex vitro to a 1:1 (*v*/*v*) peat-perlite substrate acclimatized to a high rate of 92 or 96%, respectively, retaining the characteristics of mother plants (Figure 2c,d). Ex vitro acclimatization of *S. tomentosa* was highly successful, as reported for many other medicinal Lamiaceae herbs [40–47,54], excepting *S. officinalis* that was acclimatized at lower (75%), but still satisfactory percentage [38]. Plantlet acclimatization percentage was not affected by microshoot origin in some woody species [52,79], which were acclimatized ex vitro at high survival rates (>80%) independently of their origin. However, morphological differences in produced shoots, regarding node number, compact plant shape, leaf number or dry weight have been reported between plantlets of juvenile or adult origin [52,67].

4. Conclusions

A complete micropropagation protocol was developed for *S. tomentosa* for the first time.

At the establishment stage of the cultures, explant origin (greenhouse adult plants or in vitro seedlings) and type (shoot tip or single node) mainly affected the percentage of hyperhydrated shoots produced, which was higher in explants excised from in vitro seedlings and single node explants compared with explants from greenhouse adult plants and shoot tip explants, respectively.

At the multiplication stage, high concentrations of BA (0.8–3.2 m L^{-1}) reduced the explant response for shoot production as well as shoot length and increased the number of shoots per responding explant, although many shoots were hyperhydrated especially in explants of seedling origin.

Highest multiplication indexes of normal shoots (not hyperhydrated) were produced in the hormone-free medium or the medium with low (0.4 mg L^{-1}) concentration of BA and by shoot tip explants originated from greenhouse adult plants.

Microshoots rooted more efficiently (70–78%) on MS medium either hormone-free or with the lowest concentration (0.1 mg L^{-1}) of IBA tested, regardless of whether it was full or half strength.

Ex vitro acclimatization of rooted microshoots was highly successful (92–96%) on peat-perlite (1:1, *v*/*v*) substrate.

We believe that the micropropagation protocol developed for *S. tomentosa* will contribute to the promotion of the species in the floriculture and pharmaceutical industry and will be a tool for breeding programs targeting high value products.

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