

Article

Protocol for In Vitro Propagation of *Salix acmophylla* (Boiss.). Studies on Three Ecotypes

Maurizio Capuana ^{1,*}, Werther Guidi Nissim ^{2,*} and Joshua D. Klein ^{3,*}

¹ Institute of Biosciences and Bioresources, Consiglio Nazionale delle Ricerche, Via Madonna del Piano 10, 50019 Florence, Italy

² Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza 3, 20126 Milano, Italy

³ Department of Natural Resources, Institute of Plant Sciences, Agricultural Research Organization, The Volcani Center, P.O. Box 15159, Rishon LeZion 7505101, Israel

* Correspondence: maurizio.capuana@ibbr.cnr.it (M.C.); werther.guidinissim@unimib.it (W.G.N.); vjosh@agri.gov.il (J.D.K.)

Abstract: *Salix acmophylla* Boiss. has a traditional role in several regions of the Middle East and the Indian subcontinent. As with many other woody species, the development of methods for fast propagation and ex situ conservation of this species is needed. We describe for the first time a micropropagation protocol of three *S. acmophylla* ecotypes. The best results for shoot proliferation were obtained by culturing the shoot explants on Woody Plant Medium (WPM) containing benzyladenine (BA) 5 µM, but it was also observed that proliferation can be further enhanced by the separation of the shoot tip from the underlying internodes, followed by their respective culture on BA- or activated-charcoal-containing medium. Thidiazuron or zeatin did not enhance shoot proliferation. The rooting of shoots occurred spontaneously, but for the ecotype with a lower propensity for rooting, treatment with 5 µM indolebutyric acid (IBA) increased rooting percentage. Genetic differences were evident in rooting success, but not in shoot development of the tested ecotypes in response to in vitro cultural conditions.

Keywords: growth regulators; micropropagation; rooting; shoot proliferation; willow



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1. Introduction

Willows (family Salicaceae; genus *Salix*) comprise more than 450 species distributed from subtropical to boreal climates. In recent decades, willows have become an important biomass crop, as well as being used for various other purposes including windbreaks, river-bank stabilization, and riparian filters [1–4]. Willows also have important ornamental characteristics and therefore are used in parks and gardens [5] and also in farming landscapes [6]. In the framework of the increasing interest in the exploitation of woody plants in phytoremediation [7] several *Salix* species have been investigated, e.g., [8–10].

Although most *Salix* species can be propagated by rooting of cuttings, tissue culture techniques are a valid alternative for different goals, such as clonal propagation of difficult-to-root species or clones, speeding up breeding programs, applications in genetic engineering, and simplification in the international exchange of plant material [11–13].

The first protocols for the in vitro propagation of willows date back to the early 1980s. Bhojwani [14] reported a micropropagation method for the hybrid *Salix matsudana* Koidz. x *S. alba* L., Chalupa [15] a protocol for the micropropagation of *S. alba*, *S. fragilis* L. and *S. viminalis* L., and Dhir et al. [16] for *S. babylonica* L. Bergman et al. [17] published a study on the effect of different concentrations of N⁶-benzyladenine on shoot differentiation in different willow species and hybrids. In vitro propagation protocols have also been developed for *S. exigua* L. [18], *S. caprea* L. [19], the hybrid *S. fragilis* L. x *S. lispoclados* Dode [20], *S. tarraconensis* Pau [21], *S. nigra* L. [22], *S. pseudolasiogyne* H.Lev. [23], *S. capsica*

Pall., *S. triandra* L. and *S. purpurea* L. [24], *S. tetrasperma* Roxb. [25], *S. hastata* L., *S. lapponum* L. and *S. matsudana* [26]. These studies mostly examined the use of cytokinins such as benzyl-adenine (BA), 2-isopentenyl-adenine (2iP), kinetin (Kin) and thidiazuron (TDZ), which were tested for shoot multiplication, often singly and, in some cases, in combination with auxins. Willows also have been cultured in liquid medium [22] and in temporary or continuous immersion systems [27]. Synthetic seeds were obtained by encapsulation of nodal segments containing axillary buds of *S. tetrasperma* by Khan et al. [28]. For the long-term conservation of different willow species, Towill and Widrechner [29] tested a direct bud cryopreservation of selected genotypes.

Willows have also been the subject of studies on genetic transformation; after the first studies in which Vahala et al. [30] produced transformed callus of a number of *Salix* species, only in 2013 did Yang et al. obtain stable transformants, in *S. matsudana* [31].

Salix acmophylla Boiss. is a medium-sized, deciduous tree that can reach 9 m in height. It is native to parts of the Middle East and the Indian subcontinent, where it has been successfully planted in the plains, usually along water courses [32]; sporadically, it can be found in Turkey [33]. It can also be adapted to barren lands and cold desert environments [34].

The main uses of this willow species are, traditionally, for fuel, fodder, agriculture implements, furniture, and, more recently, also for environmental preservation and erosion control [32]. *S. acmophylla* is also grown on about 40 ha on specialized farms in Israel for ritual use in the Jewish harvest festival of Sukkot (Leviticus 23:40). The species has been studied for its potential use in phytoremediation [35,36] in semi-arid climates, given its wide-ranging natural distribution and its demonstrated tolerance to heavy metals and salinity [37]. Recently, *S. acmophylla* irrigated with pre-treated municipal wastewater was successfully used as green fodder for goats, providing specific chemicals that have high potential value in maintaining animal health [38,39]. Moreover, *S. acmophylla* is considered medicinally active as a febrifuge and is traditionally used for the treatment of abscesses, ulcers, rheumatism, and chronic dysentery [40]. As with many other medicinal plants, this species is threatened in its native area due to anthropogenic activities including deforestation and overgrazing [41]. *S. acmophylla* is uniquely distant genetically from other willows, as demonstrated by the screening of 22 primer combinations through the analysis of sequence-related amplified polymorphism (SRAP) in eight *Salix* species [42]; in this study, *S. acmophylla* appeared well separated from other willow species such as *S. alba*, *S. triandra*, *S. babylonica*, *S. matsudana* and was close only to *S. excelsa* S.G.Gmel. However, Muklada et al. [39] found that ecotypes of *S. acmophylla*, sourced from more than 40 geographically distinct sites in both Israel and Jordan, resolved into three genotypes. Two of the genotypes were natural crosses between *S. acmophylla* and *S. alba* or another unidentified *Salix*, while the third, which was the most common, was pure *S. acmophylla*.

S. acmophylla can be grown from seed or propagated by rooting of cuttings [43]. Its limited nature range must be considered in how to protect the species, therefore developing methods for propagation and ex situ conservation are desirable.

This is the first study aimed at the definition of a protocol for the micropropagation of *S. acmophylla*, through a series of experiments that study the different phases of the in vitro propagation cycle, applied to three different ecotypes.

2. Materials and Methods

2.1. Plant Material

Shoots of three *S. acmophylla* ecotypes were collected in Israel from three sites: Darom 4 (referred to hereafter as Darom), Adom, and Mea She'arim, characterized by different heights and precipitation. Darom (DA) originates in the northern Negev desert (31°45' N, 34°80' E, 250 m a.s.l.), with an average of 300 mm rainfall per year, while Adom (AD) and Mea She'arim (MS) are from Motza (31°80' N, 35°16' E, 600 m a.s.l.) and Jerusalem (31°79' N, 35°22' E, 754 m a.s.l.), respectively, both with 520–540 mm rainfall per year. Plant material (30 cm long cuttings of stem tissue) was harvested from potted plants originating

from trees grown after collecting campaigns in 2003 (Adom and Mea She'arim) and 2010 (Darom), and stored at 2 °C for 24 h.

2.2. Culture Conditions

To begin *in vitro* propagation, small pieces of stem (about 2.0 cm long) bearing one bud were excised and sterilized in 70% ethanol solution for 1 min, with a rinse in sterile distilled water, followed by 20 min soaking in 1.8% sodium hypochlorite solution and three rinses in sterile distilled water.

These excised shoots, after a new minimal cut at their base, were placed in a polar orientation in 175 mL glass vessels ("baby food jars" Sigma-Aldrich[®], Milano, Italy) containing 25 mL culture medium (5 segments per vessel). The testing media contained 30 g·L⁻¹ sucrose and 7.2 g·L⁻¹ agar (B&W, Reggio Emilia, Italy) as a gelling agent. The pH was adjusted to 5.8 before autoclaving at 121 °C and 108 kPa for 20 min. Cultures were incubated in a growth chamber at 23 ± 1 °C, with a 16 h light photoperiod supplied by Osram cool white fluorescent lamps (light intensity: 60 μmol m⁻²s⁻¹). These environmental conditions were also used for the subsequent experiments.

2.3. Axillary Shoot Proliferation

The first test was carried out to compare the two basal media most commonly used for willows: MS (Murashige and Skoog) [44] and WPM (Woody Plant Medium) [45]. The two formulations were tested after adding N⁶-BA 5 μM to the media. In this preliminary test, six shoots for each ecotype and for each treatment were used.

Subsequently, different cytokinin and cytokinin-like growth regulators were tested with WPM for the proliferation phase: benzyladenine (BA, 1 or 5 μM), zeatin (ZEA, 0.5 or 1 μM), and thidiazuron (TDZ, 1 or 2 μM) plus a control without plant growth regulators (PGR). Three replicates with a total of 18 explants for each ecotype and for each treatment were tested.

After proliferation, the isolated shoots were repeatedly subcultured on WPM containing 0.2 mg·L⁻¹ activated charcoal (AC, BDH[®], England) in order to stimulate the development of buds into shoots after the PGR treatments. In these trials, the number of proliferated shoots was recorded after PGR treatment and one subculture on AC-containing medium.

The results of this test led us to a second experiment on the proliferation/elongation phase by comparing two procedures: a "standard" scheme consisting of the aforementioned procedure carried out with the most effective PGR treatments (scheme "A"), and a procedure in which the 2 cm-long shoot tip was subcultured on AC-containing WPM and the underlying internode(s) were treated with the PGR selected after the previous test (scheme "B"). For this experiment, we used five shoots of each of the three ecotypes previously multiplied on BA (5 μM)-containing WPM and then subcultured for two months on AC-containing medium in order to minimize the possible residual effect of the hormonal treatment. In this test, the total number of developing shoots was recorded after each of the four culture cycles.

2.4. Rooting and Acclimatization

For the rooting trial, we used plantlets of the three tested ecotypes proliferated on BA(5 μM)-containing WPM and then repeatedly subcultured on AC-containing medium. We compared plantlets cultured on either AC (0.2 mg·L⁻¹)-containing WPM (control) or after a 3 wk culture on WPM supplemented with 5 μM indolebutyric acid (IBA). Data on rooting were recorded on 24 samples × ecotype × treatment after a subsequent 4 wk subculture on AC-containing medium. We recorded the number of rooted plants (shoots with at least 1 emerging root longer than 2.0 mm), the number of roots, and the length of the roots.

After rooting, the plantlets were removed from the culture medium and, after gently washing the root system under running tap water, were transferred to 450 mL plastic pots containing autoclaved soil (a 3:2:1 mixture of garden soil, peat, sand). The plantlets

were initially grown in an environmentally controlled cabinet, at 23 ± 2 °C, light intensity $60 \mu\text{m}^{-2}\text{s}^{-1}$ with photoperiod $16 \text{ h}\cdot\text{d}^{-1}$, and relative humidity progressively decreasing from 90 to 50% over four weeks. Plants were then transferred outdoors, to a shaded area of the nursery.

2.5. Statistical Analysis

All collected data were analyzed for normality using the Shapiro–Wilk test and log transformation was performed on variables not showing a normal distribution. For shoot proliferation, number of formed roots and root length, a two-way analysis of variance test, followed by a posthoc Tukey’s HSD test, was used to determine significant differences between treatment group means with a threshold of 0.05. A χ^2 test was performed for the categorical variable of rooting percentage ($p < 0.05$).

3. Results

3.1. Effect of PGRs on Shoot Proliferation

WPM was more effective than MS in our preliminary trial, leading to a higher multiplication rate (MR, mean number of proliferating axillary shoots per explant) of 1.05 for WPM, compared with 0.78 for MS. Some chlorosis was observed in the plantlets cultured on MS medium (data not shown). The WPM was therefore used for all subsequent experiments. In the proliferation test, the MR was generally low, with values ≤ 0.5 for shoots treated with TDZ or ZEA. Better results were obtained after the BA treatment, with the best performance recorded for the 5 μM treatment: for all three tested ecotypes, the resulting MR was always slightly above 1.0, reaching 1.25 for the MS ecotype (Table 1). There were no significant differences between ecotypes in their response to the treatments for shoot proliferation. Shoots cultured in media containing either TDZ or ZEA were frequently vitrified and had undesirable basal callus production.

Table 1. Multiplication rate (MR) of three *S. acmophylla* ecotypes. Data are mean number of proliferating axillary shoots recorded after PGR treatment and subsequent growth on AC-containing medium.

Treatment (μM)	Shoots per Explant								ANOVA <i>p</i> Values						
	Mea Shearim		Adom		Darom		Treatment Means		Ecotype (E)	Treatment (T)	ExT				
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM							
Control	0.00	(0.00)	d	0.17	(0.11)	d	0.08	(0.08)	d	0.08	(0.04)	d			
BA1	0.83	(0.11)	b	0.58	(0.15)	b	0.83	(0.21)	b	0.74	(0.09)	b			
BA5	1.25	(0.13)	a	1.08	(0.08)	a	1.00	(0.12)	a	1.12	(0.06)	a			
ZEA 0.5	0.42	(0.15)	c	0.25	(0.13)	c	0.42	(0.15)	c	0.41	(0.08)	c	0.3721	<0.001	1.9867
ZEA 1	0.50	(0.15)	c	0.33	(0.14)	c	0.50	(0.15)	c	0.44	(0.09)	c			
TDZ 1	0.42	(0.15)	c	0.25	(0.13)	c	0.33	(0.14)	c	0.33	(0.08)	c			
TDZ 2	0.33	(0.13)	c	0.36	(0.15)	c	0.27	(0.13)	c	0.31	(0.08)	c			
Mean	0.54	(0.12)		0.43	(0.13)		0.49	(0.14)		0.49	(0.07)				

Values are the mean for each treatment \pm SEM ($n = 18$). A 5% significance level was adopted for identifying significant treatment effects according to Tukey’s HSD test. Different lowercase letters in each column indicate significant differences among hormonal treatment. BA—benzyladenine; ZEA—zeatin; TDZ—thidiazuron.

Shoots grew quickly after transfer from PGR treatment to the AC-containing medium (Figure 1a): in one subculture, lasting 4 weeks, the shoots produced two to three internodes, thus allowing the sectioning of explants for the following subculture. We therefore performed a parallel test on shoot growth and proliferation with repeated subculturing. In this treatment (Figure 2, treatment “B”), we observed that starting from the initial batch of 5 explants per ecotype, by the fourth subculture we obtained 110, 102 and 112 explants for the ecotypes MS, AD and DA, respectively. These results were significantly greater than those obtained with treatment “A”, in which just under 80 collectable explants resulted

from all the tested ecotypes. The data presented in Figure 2 suggest an acceleration of this gap for subsequent subcultures.



Figure 1. (a) In vitro shoot development of *S. acmophylla* on AC-containing WPM; (b) rooted plantlets of *S. acrophyll*, two weeks after acclimatization.

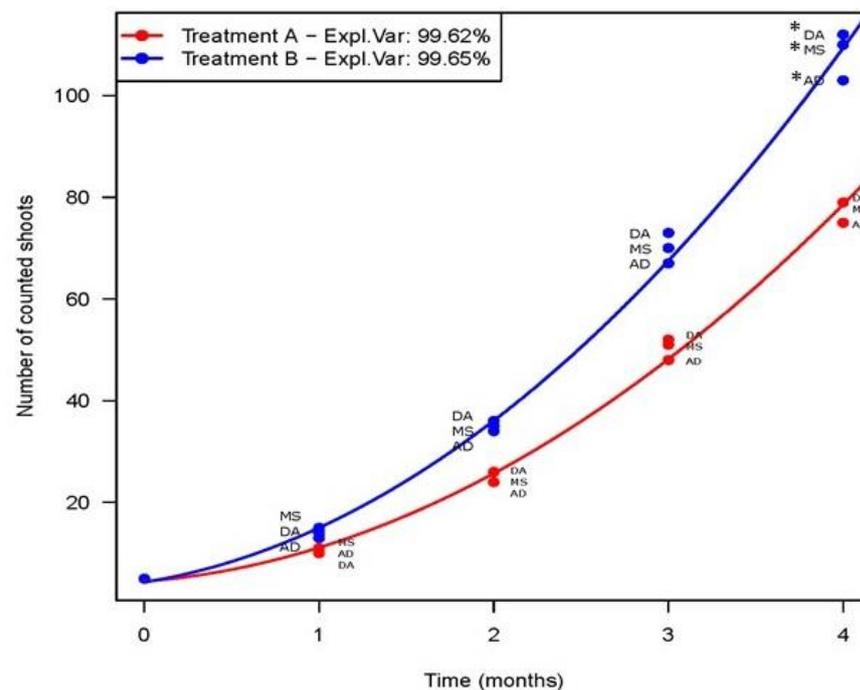


Figure 2. Predictive growth model of shoot multiplication. Data are cumulative number of shoots observed after the first, second, third and fourth subcultures from an initial lot of five shoots according to two different procedures of cultivation of three *S. acmophylla* ecotypes. MS: Mea Shearim; AD: Adom; DA: Darom. Treatment A: Continuous subculturing on BA-containing medium; Treatment B: Isolation of the shoot apex (first internode) and its culture on activated charcoal (0.2 mg L^{-1})-containing medium and culture of the underlying internode(s) on BA ($5 \mu\text{M}$)-containing medium. Values were analyzed by χ^2 test; asterisks indicate a 5% significance level between treatments within ecotypes.

3.2. Rooting and Acclimatization

Rooting occurred spontaneously in all ecotypes during the proliferation phase (from 25 to 40% of shoots, depending on the ecotype—data not shown). The AD shoots cultured

on WPM with IBA had a 29% increase in rooting compared to control with 0.2 mg L^{-1} AC, while, with the DA and MS ecotypes, treatment with IBA increased rooting by an average of 11% compared to controls (Table 2). The mean number of emerging roots ranged from 3.1 to 3.8 per explant, with a significant improvement after the IBA treatment only for the AD ecotype. The mean root length was around 40 mm for the DA and MS ecotypes in both the control and IBA-treated shoots; in the AD ecotype, it increased from 29.4 (control) to 36.1 (treated). Plantlet acclimatization (Figure 1b) was around 90% for all three ecotypes.

Table 2. Rooting percentage, mean number of formed roots and mean root length in cultured shoots of three *S. acmophylla* ecotypes subjected to either IBA $5 \mu\text{M}$ or control treatment (AC 0.2 mg L^{-1}). MS—Mea Shearim; AD—Adom; DA—Darom.

Ecotype	Treatment	Rooting (%)	Roots (n)		Root Length (mm)	
			Mean	SEM	Mean	SEM
MS	Control	83.3 a	3.8	(0.17) a	39.7	(0.36) a
	IBA	95.8 a	3.8	(0.14) a	40.4	(0.34) a
	Mean	89.5 A	3.8	(0.16) A	40.1	(0.35) A
AD	Control	58.3 a	3.1	(0.14) b	29.4	(0.58) a
	IBA	87.5 b	3.8	(0.13) a	36.1	(0.49) b
	Mean	72.9 B	3.4	(0.13) B	32.7	(0.54) B
DA	Control	87.5 a	3.6	(0.17) a	38.2	(0.58) a
	IBA	95.8 b	3.5	(0.13) a	40.0	(0.34) a
	Mean	91.6 A	3.5	(0.15) B	39.1	(0.42) A
Treatment Means	Control	76.4 b	3.5	(0.16)	35.8	(0.51)
	IBA	93.0 a	3.7	(0.13)	38.8	(0.39)
ANOVA	Ecotype (E)			0.05		<0.001
<i>p</i> -values	Treatment (T)			0.08		0.007
	ExT			0.03		<0.001

Different letters in each column indicate significant differences among hormonal treatment.

Rooting percentage was analyzed by a χ^2 test. Values for number of roots and root length, analyzed by Tukey's test, are the mean for each treatment \pm SEM ($n = 24$). A 5% significance level was adopted for identifying significant treatment effects according to χ^2 test and Tukey's HSD. Different capital letters denote a significant difference between willow clones. Different lowercase letters in each column indicate significant differences between treatments.

4. Discussion

4.1. Moderate Effectiveness of PGR Treatments in the Shoot Proliferation

Our results further confirmed the difficulties encountered in obtaining a highly efficient in vitro propagation system for *Salix* species [19,21,46]. In vitro cultured willows are generally characterized by strong apical dominance, in which axillary bud growth is greatly inhibited by the shoot apex or the first axillary bud [21]. Despite the positive effect on shoot proliferation often reported for the application of zeatin [47–49] or TDZ [50–52] to other woody species, neither of these two plant growth regulators were effective with *S. acmophylla*.

Only $5 \mu\text{M}$ BA provided reasonable results, but the MR of nearly 1.0 is probably low for commercial in vitro propagation. Despite low bud proliferation, the administration of this limited dose of BA did not completely limit apex growth, allowing for continuous, if reduced, shoot development. Bergman et al. [17] demonstrated, at low concentration, a positive effect of BA on axillary shoot development, but not on adventitious bud formation, with five species and clonal hybrids of *Salix*.

Following a different regeneration procedure, involving organogenesis, callus induction in *S. exigua* was obtained using WPM supplemented with BA and 2,4-D, and shoot regeneration was induced subsequently following BA treatment [18]. The application of

0.2 mg L⁻¹ BA also led to shoot multiplication from ovary culture in a hybrid willow (*Salix fragilis* × *S. lispoclados*) [20].

4.2. An Alternative Procedure Makes the Proliferation Phase More Effective

The *S. acmophylla* ecotypes available for this experiment had rather low bud proliferation rates, but apical bud development was vigorous, suggesting that this fast growth might optimize the multiplication procedure. The isolation of internodes during continuous shoot growth in a medium with a moderate amount of BA led to fast sprouting of the axillary bud and its subsequent development into a new vigorous shoot. These observations are similar to those of Gebhardt [53] on *S. viminalis*, *S. fragilis*, *S. pentandra* and others, that reported good shoot development and rooting occurring on a medium without growth regulators, but even browning of shoot tips on a cytokinin-containing medium. Chalupa [15], in his first observations on willow micropropagation, concluded that it is possible to rapidly propagate these species by repeated division of the stem into nodal segments, without additional hormones.

Our parallel observations on shoot growth and proliferation according to two different culture methods led us to consider the usefulness of differentiating the cultivation of the apices from that of the underlying internodes, similar to the conclusions of Chalupa [15].

4.3. Positive Results in the Rooting and Acclimatization Phases

Rooting took place mostly spontaneously during the shoot proliferation and elongation phases, as Amo Marco and Lledo [21] with *S. tarraconsensis*, Liesebach and Naujoks [46] with *S. caprea*, Lyyra et al. [22] with *S. nigra*, Park et al. [23] with *S. pseudolasiogyne*, and Regueira et al. [54] with *S. viminalis* have reported. However, Khan and Anis [55] reported no rooting in *S. tetrasperma* after culture on auxin-free basal medium, identifying as a possible cause the residual effect of TDZ, considered detrimental for in vitro rooting [56].

Despite the pronounced spontaneous rooting that we observed, we decided to carry out a simple rooting trial, especially since the AD ecotype previously rooted poorly compared to the other two ecotypes. The high spontaneous rooting of ecotypes MS and DA was not further improved, while IBA treatment significantly enhanced rooting in the AD ecotype, to a final rooting percentage comparable to that of the other two ecotypes. IBA is effective in enhancing rooting in other *Salix* species, such as *S. tetrasperma* [28], and also *S. dasyclados*, *caspiica*, *triandra*, *purpurea*, and *viminalis*, as well as hybrids [24]. Amo-Marco and Lledo [21], after a test using three auxins (IBA, IAA and NAA) at different concentrations, recorded a high rooting percentage (90–100%) on all the media tested, including control, but observing a negative effect with NAA, with the outgrowth of abnormally thick roots and excessive callus production at the base of the shoots. In *S. acutifolia* Willd., however, the best rooting performances were obtained after 8 weeks on media containing NAA 0.2 mg L⁻¹, reaching 97 and 93% rooting on MS and WPM, respectively [57].

IBA treatment enhanced the number of roots and their mean length, but only for the AD ecotype (Table 2). A positive effect of 0.5 µM IBA on the same parameters was observed on *S. tetrasperma* by Khan et al. [25].

All three *S. acmophylla* ecotypes were sensitive to a rapid decrease in ambient humidity in vitro, as observed in a preliminary test on plantlet acclimatization which led to shoot apex wasting and severe browning of leaves (data not shown). However, plantlet acclimatization under the adopted experimental conditions was successful, with no substantial differences for the three tested ecotypes and at percentages comparable to those observed for other willow species [21,25].

4.4. Differences Found among the Tested Ecotypes

We found different ecotype performance only during the rooting phase. Several studies indicate genotypic differences in the behavior of in vitro shoot culture of willows [17,46,58], as well as in *Populus*, a closely related genus [59].

During the course of our experiments, colleagues in Israel, Jordan, and the US genotyped more than forty *S. acmophylla* ecotypes from locations in Israel and Jordan [39]. They found that while there is a large population of pure *S. acmophylla* in both countries, and there are two additional hybrid populations. One is found in both countries and is a cross of *S. acmophylla* and *S. alba*. The other population is a hybrid of *S. acmophylla* and an as-yet unidentified *Salix* species. Adom (AD) is pure *S. acmophylla*. Mea Shearim (MS) and Darom (DA), despite originating from sites more than 80 km apart, are both from a genotype that is approximately 60% *S. acmophylla* and 40% a currently unidentified *Salix* species. Mea Shearim, Adom and Darom are similar in leaf and stipule morphology, and are all resistant to *Melampsora* rust, which severely affects the other hybrid genotype. However, pure *S. acmophylla* trees, although rich in antioxidant compounds [38,39], with more colorful foliage and bark than the hybrids, are significantly smaller and weaker than the hybrids and have a shorter life-span (Klein, unpublished).

The ecotypes investigated in the current research turn out to represent their respective genotypes very well: Adom has leaves and bark that are redder than those of Mea Shearim and Darom, and its smaller form and weaker growth in vitro is reflected in its growth habit in outdoor plantings as well. On the other hand, these very characteristics of Adom emphasize the need to develop a propagation protocol that can ensure ease of production of this genotype for aesthetic, environmental and agricultural [38] purposes.

5. Conclusions

We developed a successful in vitro propagation protocol for three *S. acmophylla* ecotypes. The use of nodal explants allows an acceptable multiplication rate, achievable by either shoot segmentation or bud proliferation. The transfer of shoots to PGR-free medium containing activated charcoal is the quickest procedure to get rooted plantlets from the two tested ecotypes with high rooting potential, while the ecotype with a lower rooting potential benefited from IBA treatment. Plantlets are then easily acclimatized and grown in a greenhouse. The multiplication of plantlets was obtained without an intervening callus phase, thus avoiding the risks of somaclonal variation. In vitro propagation of *S. acmophylla* had not previously been studied. Our initial results lead us to ascribe this species to the group of tree species such as *S. pseudolasiogyne* [23] that are difficult to propagate in vitro without amendments to the medium. Future studies could be directed to improving the shoot multiplication rate, with the aim of making the procedure practical for commercial applications.

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