

Review

# Propagation of Juniper Species by Plant Tissue Culture: A Mini-Review

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**Abstract:** The genus *Juniperus* (of the Cupressaceae family) is the second most prevalent group of conifers on Earth. Juniper species are widely dispersed in the Northern Hemisphere, in Europe and Asia, and in Africa and Central America. Juniper species are resistant to dry climates and can adapt to difficult environmental conditions. Most juniper species are important in both ecological and economic terms. However, today, many forests in which junipers occur are being reduced in size due to both natural causes (fires, for example) and human activity (uncontrolled exploitation of forests, etc.). Also, climate changes may have adversely affected the range of populations of different juniper species. For this reason, some juniper species are now categorized as rare or endangered, and require immediate protective action. Therefore, there is an urgent need to develop effective strategies for ex situ conservation, including reliable procedures for *Juniperus* sp. reproduction for future reintroduction and restoration programs. The conservation strategies used until now with traditional forestry techniques (seed propagation, rooted cuttings, grafting) have not been satisfactory in many cases. Thus, increasing attention is being paid to the possibilities offered by in vitro culture technology, which enables the conservation and mass clonal propagation of different coniferous tree species. In this mini-review, we summarize the current state of knowledge regarding the use of various methods of the propagation of selected *Juniperus* species, with a particular emphasis on in vitro culture techniques.

**Keywords:** germination in vitro; micropropagation; organogenesis; somatic embryogenesis; juniper; endangered species

## 1. Introduction

*Juniperus* L. is a genus of conifers belonging to the family Cupressaceae. After the pines, it is the most common group of evergreen shrubs and trees on Earth, and includes approximately 67 species [1]. Junipers grow in temperate and subpolar climates in Eurasia and North America, and in the mountains in the tropics. For example, Phoenicean juniper *Juniperus phoenicea* is native to some regions of the Mediterranean Basin, Canary Islands and North Africa [2]. Greek juniper *J. excelsa* forms the second largest forest in the world and is the oldest living species on earth [3]. In turn, Portuguese prickly juniper *J. navicularis* is an endemic dioecious shrub from the Plio-Plistocene transition sands of the west Portuguese coast line [4]. In general, *Juniperus* species are resistant to dry conditions and are adaptable to stressful environments [5,6]. A small tree of *J. phoenicea* grows successfully in areas with persistent drought and arid climate with high temperature ranges [7]. Junipers play a very important ecological role, protecting the soil from erosion [8]. In the dry regions of the Iran countryside, eastern juniper *J. polycarpos*, which occurs usually at high altitudes, over 1000 m on the sandy rock soil, prevents soil erosion, windy erosion and desert greening [9]. In many countries, juniper wood has economic value and is used for the production of lead pencils, the construction of buildings and a variety of outdoor structures, etc. [10,11].

Moreover, juniper berries are the source of oil for the production of several pharmaceutical preparations and for flavoring gin [12]. Some extractions are (or may be) also used in the treatment of infections, fungal and contagious diseases such as colds, gynecological diseases, tumors and diabetes [6,13]. Extractives of Chinese juniper *J. chinensis* leave contain podophyllotoxins which have strong antileukemic and tumor-inhibitory effects [13]. Oils isolated from the leaves of *J. phoenicea* show inhibition effects against bacteria and fungi [7]. Some juniper species are cultivated as ornamental plants [14] for their diverse habits, density, color and leaf length and position.

Currently, the natural habitats of some *Juniperus* sp. (*J. chinensis*, *J. excelsa*, creping juniper *J. horizontalis*, cade juniper *J. oxycedrus*) are greatly reduced [3,15]. The main reasons are overexploitation for timber and fuel wood, fire, the poor propagation of some species from seed [5,16–18], pest-infected cones [19] and the very slow growth rate of trees [20,21]. Recently, climate changes may have also adversely affected the range of populations of different juniper species. According to literature data a few juniper species are considered as rare or endangered [4,17,18,22,23]. There are: *J. navicularis* (an endemic and rare species in Portugal), Spanish juniper *J. thurifera* (a rare, endangered species in Algeria), Canary Islands juniper *J. cedrus* (endangered species, native to the Canary Islands, Spain), *J. excelsa*, and common juniper *J. communis*. Although *J. excelsa* is widely distributed in the drier forests of East Africa, south and central Asia and south eastern Europe, it is on the verge of extinction [18]. In turn, some *J. communis* populations in certain insular areas or in Turkey are considered under risk as a result of low regeneration capacity [23]. Therefore, attempts have been made to restore juniper forests based on the different techniques of sexual and asexual propagation [19]. However, the sexual propagation of juniper is very often limited mainly due to their low seed production, low germination rate, deep physiological dormancy and decreased viability of the seed embryos [6,17,18]. Lower seed quality results also from insufficient pollination and from infections of cones by pests [19,20,24]. Another problem for this propagation strategy is the elevated level of heterozygosity and the extended cycle of seed production, which in juniper is up to eight years [25]. A very low potential of regeneration from seeds was reported for such species as: *J. excelsa*, *J. chinensis*, *J. horizontalis*, *J. navicularis*, *J. phoenicea*, *J. polycarpos*, and red juniper *J. virginiana* [3,4,9,26]. Also the problem with the asexual propagation of *Juniperus* species by the rooting of vegetative cuttings has not been solved for many of them thus far [6,27]. The success in cutting rooting of juniper branches was often reported to be at less than 30% [9]. For example, only 24% of the branch cuttings of African juniper *J. procera* obtained from 1.5–2-year-old stock plants rooted 32 weeks after treatment. In this juniper species the age of stock plants was the most important factor controlling rooting of the cuttings. Such low success of rooting makes it impossible the large scale propagation of the tree [27]. Generally, conventional breeding of woody trees is a slow and difficult process [25]. Therefore, micropropagation may be an alternative reproduction method for this group of plants [12]. It is a highly efficient method of vegetative reproduction used in the breeding of many shrubs and tree species [28,29], including rare and endangered species [30–32]. Currently, in vitro techniques are commonly used in biotechnology and mass plant production [33,34], complementing conventional plant propagation and contributing to plant quality improvement and mass propagation [33]. Micropropagation is widely used primarily in horticulture and agriculture, but it has only been used to a limited extent in forestry [34].

The first work on the reproduction of juniper by in vitro cultures was performed by Javeed and co-workers in 1980 [35]. Since then, few studies have been published on this subject [2,3,12,16,18,22,36], and only single publications have reported the success of this method. Therefore, research on the micropropagation of junipers should be intensified because in many cases, the in vitro method may be the only alternative allowing the cloning of increasingly rare and shrinking populations of these woody plants. The development of new strategies for the reproduction of junipers based on in vitro techniques may be particularly important today in the face of climate change, an additional threat to natural forest ecosystems.

In this review, attempts at the micropropagation of several juniper species over the last 30 years are discussed. Our objective is to consolidate and summarize existing knowledge in this area and to discuss the future scope of improvements.

## 2. Seed Germination

All juniper species have sexual reproductive capacity, but the number of seeds they produce is very variable and unstable. A large quantity of the produced seeds are empty, damaged or dead. *J. thulifera* may produce more than 60% empty seeds [37]. *J. polycarpos* seed infestation decreased the seed viability by 67% and germination capacity by 78% [38]. Studies have shown that some species, such as *J. communis*, *J. sabina* and *J. thurifera*, produce a very small number of viable seeds [37,39]. For example, the subspecies *J. sabina arenaria* produces very small cones (approximately 4–6 mm in diameter), which contain only 2–6 seeds [39]. A large proportion of the obtained seeds are also anatomically undeveloped. The low number of properly developed seeds is one of the reasons limiting the sexual reproduction of these coniferous plants [11,20]. Under the current climate change conditions and strong anthropogenic pressure, when the range of juniper populations is increasingly being reduced, this state of affairs is very worrying; the low germination frequency of juniper seeds is also a significant problem [7,40]. For example, it was found that very low *J. polycarpos* plant production was due to the decreased seed germination level [9]. In several *Juniperus* species, the main reason for this is the deep physiological dormancy of the seeds [41], which prevents their germination and is difficult to break. Attempts made by scientists to improve the germination efficiency of seeds of particular species using different techniques were not always successful. The proposition of using of unripe juniper seeds proved to be ineffective [42]. Negative results were obtained after treatment of *J. cedrus* seeds with concentrated nitric ( $\text{HNO}_3$ ) or sulfuric acid  $\text{H}_2\text{SO}_4$ , and after cold stratification for 30–60 days at 4–5 °C [17]. Also attempts to germinate the seeds of *J. oxycedrus* in greenhouse conditions using intact seeds and seeds without testa did not resolve the problem of the low germination capacity of the seeds [10]. However, subsequent studies have obtained positive results for some juniper species [40,43–46]. For better germination of *J. procera* seeds it was recommended to employ cold-moist stratification for six weeks [45]. However, it was found that the success was dependent upon genetic and environmental components, which strongly affect the degree of dormancy in this juniper species. The idea of smoke treatment to overcome the photodormancy in *J. procera* seeds turned out to be ineffective in these studies. The improved germination response of *J. procera* seeds was observed after their exposure to the narrow range of red/far red light and a constant temperature of 20 °C [44]. In turn, warm-cold stratification of *J. communis* seeds carried out at 15 °C/3 °C for 14 + 12 weeks increased the germination capacity and seedling emergence in this species [43]. Seeds of *J. sabina* reacted similarly to this treatment; however, the warm stratification was more effective at 20 °C [46]. In these studies, the seed germination rate was improved from 25% to 46%. Recently, research conducted on *J. polycarpos* confirmed the effectiveness of the warm-cold stratification of seeds at 20 °C/1 °C for 16 + 12 weeks in junipers [40]. In this case, the germination capacity was improved from 8% to 72%. The authors did not obtain satisfactory results after the treatment of seeds with gibberellic acid ( $\text{GA}_3$ ) and 6-benzyladenine (BA) alone or in combination with cold stratification.

The cited data show that breaking the dormancy of juniper seeds based on the stratification method is the correct approach, and the more so, when other methods rarely provide a positive result. Therefore, many more studies in this area, including studies of other juniper species for which seed germination is limited by physiological dormancy, should be undertaken.

## 3. Seed Germination In Vitro

One of the reasons for seed dormancy is the presence of inhibitors in the embryo, endosperm or testa. To eliminate the inhibitory effect of these substances, seeds without testa or isolated embryos are incubated on media under controlled, in vitro culture conditions, to stimulate the germination process. This approach has also been applied by some researchers to increase the germination rate of certain

juniper species [10,37]. A relatively low response (12%) was observed when *J. oxycedrus* seeds without testa were incubated on  $\frac{1}{3}$  strength MS, Murashige and Skoog (1962) medium [47] supplemented with 3% sucrose with or without GA<sub>3</sub>. Much more promising results were obtained under the same conditions for embryos isolated from seeds (approximately 50% germination rate).

Moreover, plants derived from isolated embryos were able to acclimatize in soil at a high level (83% and 75% for the *J. oxycedrus* subspp. *oxycedrus* and *macrocarpa*, respectively) [10]. Recently, successful germination of isolated embryos of *J. thurifera* under in vitro culture conditions was reported [37]. The highest germination rate for this rare juniper species was 80% on the solid DCR, Gupta and Durzan (1985) medium [48]. As demonstrated in the studies cited, the use of an in vitro culture technique in zygotic embryo germination may be a promising way to overcome the problems with low germination rates in junipers and to improve their propagation via seeds. Furthermore, in our opinion, the capability to germinate zygotic embryos in vitro creates the opportunity to use them as a potential source of sterile explants (shoots, buds, roots, leaves, protoplasts, etc.) for the induction of juniper cultures via various in vitro propagation techniques (organogenesis and somatic embryogenesis, for example).

#### 4. Micropagation

##### 4.1. Explant Sterilization Methods

The first factor in ensuring high-quality tissue plant cultures is proper disinfection of the starting material. In conifers, many procedures have been developed to safely introduce the plant material into in vitro conditions. In the case of *Juniperus* species micropagation, most works reported no problems with contamination when different fragments of plants were used as the initial explants [5,15–17,23,49]. Aseptic cultures were obtained for various types of explants collected from mother plants regardless of the age of the donor plant [7,9,12,36,49–51]. A high degree of sterility of explants (99%) was reported for microcuttings from mature trees of *J. navicularis* using 70% ethanol, followed by immersion in 3% commercial bleach (Domestos®, Unilever, England, UK), washing in 1% Benlate solution and finally immersion in 70% ethanol [4]. A highly sterile explant level (98%) was obtained after treatment of shoot tips originating from approximately 7-year-old trees of *J. polycarpos* based on the optimized surface sterilization protocol [9,52]. Pathogen-free explants have also been reported as a result of the treatment of young shoot tips of *J. excelsa* with 2.5% chlorine from sodium hypochlorite (NaOCl) [15]. Recently, two disinfection procedures were tested for explants of *J. thulifera* (shoots from 8- to 10-year-old trees) [22]. This study demonstrated that the type of sterilization has a decisive influence on the infection control of initial explants. After using sodium hypochlorite solution, all explants were lost. However, after immersion in a systemic fungicide mixture, followed by immersion in sodium hypochlorite solution, a very high level of sterilization was obtained. A 40% contamination level during callus initiation from leaves originating out of garden-grown plants was reported [13], but there was no information provided about the applied sterilization method. However, the best source of juniper explants for establishing a contamination-free culture is plant material derived from in vitro growing plants [16,53,54], because they do not require any sterilization procedure to be used as an explant source.

According to the literature, there are no problems with the decontamination of juniper seeds used as a source of explants for the induction of in vitro cultures [5,12,17,50]. Pinkish and yellowish microbial (bacterial and yeast) contamination of zygotic embryos of *J. excelsa* as the initial explants were reported five days after the start of culture [18]. However, the authors pointed to external conditions (the transfer and incubation of the plant material), rather than the inappropriate sterilization method, as the source of the contamination. Completely contaminated mature seed explants of *J. phoenicea* used for in vitro culture establishment were reported, although the applied method was efficient for microcuttings originating from 3-year-old seedlings of this juniper species [7]. Explants were treated with household detergent and immersed in 2.5% active chlorine from sodium hypochlorite. According to the authors, the problem with the successful disinfection of seeds of *J. phoenicea* could be associated

with their morphological features, namely, the presence of characteristic grooves along the surface of the seeds, which reduce the surface sterilization and thus enable microbes to develop with prolonged culture time.

#### 4.2. Callus Induction from Different Explants

In many plants, a callus is obtained from various types of organs or tissues. The callus induction frequency is closely correlated with the age of the explants, and the most suitable explants are usually the youngest. In coniferous species, an appropriate ratio of auxin and cytokinin should be provided to stimulate the explants for callus development. The first callus cultures in juniper were established by Javeed and co-workers [35] and by Ilashi in the first half of the 1980s [12] (Table 1). A soft, friable callus of a creamy color from 2-year-old shoot cuttings of *J. polycarpos* was obtained on MS medium supplemented with  $0.5 \text{ mg L}^{-1}$  2,4-dichlorophenoxyacetic acid (2,4-D) and  $2 \text{ mg L}^{-1}$  N6-furfuryladenine (KIN) [12]. A callus was able to further proliferate on the same medium, but no organogenesis was observed. This was probably due to the presence of auxins in the medium, which reduces the organogenesis process in conifers even if they are applied at low concentrations [55]. Recently, the possibility of inducing callus in *J. excelsa* using lateral bud cuttings taken from 8-year-old plants was demonstrated [21]. The research revealed a 73% callus induction frequency using this type of explant. Organic callus was obtained at the highest frequency on MS medium in the presence of higher concentrations of auxin 2,4-D ( $3.0 \text{ mg L}^{-1}$ ) and KIN (N6-furfuryladenine,  $0.2 \text{ mg L}^{-1}$ ). Additionally, the successful induction of *J. chinensis* callus cultures from the leaves of young trees was reported on SH, Schenk and Hilderbrandt (1972) medium [56] after application of higher concentrations of auxin 1-naphthaleneacetic acid (NAA;  $3.0 \text{ mg L}^{-1}$ ) and KIN ( $0.2 \text{ mg L}^{-1}$ ) [13]. The induced callus was white to light yellowish-brown and proliferated on the same medium for five subcultures. However, after ten subcultures, the callus lost its ability for multiplication. In turn, callus cultures of *J. virginiana* were induced using fresh leaves of three different varieties of this tree as explants [57]. The highest callus induction frequency was 88.1% for the variety 'Glauca' on SH medium containing  $3.0 \text{ mg L}^{-1}$  NAA and  $0.2 \text{ mg L}^{-1}$  KIN. The induced calluses quickly turned brownish, but the application of ascorbic acid ( $15 \text{ mg L}^{-1}$ ) to the medium as an antioxidant eliminated this phenomenon. To improve the callus growth rate, the authors proposed the application of cell suspension cultures. In these conditions, the calluses proliferated faster than in the traditional callus cultures. In conifers, the rapid loss of callus multiplication capacity under in vitro cultures is a serious problem. Consequently, it is impossible to maintain callus cultures for a longer time and to use them as a source of adventitious buds. Therefore, for some *Juniperus* species, research on the development of much more stable and efficient micropropagation techniques is necessary.

**Table 1.** Micropropagation and tissue cultures of *Juniperus* spp.

Species	Explant Type, Size and Origin	Culture Medium, PGRs and Activities	Culture Conditions	References
<i>J. polycarpos</i>	Juvenile shoot pieces			Javeed et al. 1980 (after Ilashi 1986) [35]
<i>J. polycarpos</i>	Two studies: 1) Shoot cuttings (1–1.5 cm) from 2-year-old plants and 20-year-old mature trees 2) Mature zygotic embryos	1) MS + $0.5 \text{ mg L}^{-1}$ 2,4-D + $2.0 \text{ mg L}^{-1}$ KIN (CIM) MS + $0.5 \text{ mg L}^{-1}$ 2,4-D + $1.0 \text{ mg L}^{-1}$ KIN. 4 w. (CPM) 2) $\frac{1}{2}$ or full MS + 10% CW, 5–10% sucrose. 8 w. (CIM)	No information	Ilashi (1986) [12]

Table 1. Cont.

Species	Explant Type, Size and Origin	Culture Medium, PGRs and Activities	Culture Conditions	References
<i>J. oxycedrus</i>	Leaves (0.5 cm) from shoots growing in vitro obtained from terminal shoots of 30-year-old wild trees	Modified SH + 0.11 mg L <sup>-1</sup> BA, 3% sucrose, 0.7% agar, pH 5.8. 45 d. (SIM) Modified SH + 0.05% AC without BA, 4% sucrose. 45 d. (SEM) Modified SH + 0.47 mg L <sup>-1</sup> NAA, 4% sucrose. 60 d. (RIM)	26 ± 2 °C, 16-h PP, FTL 80 μmol m <sup>-2</sup> ·s <sup>-1</sup>	Gomez and Segura (1994) [16]
<i>J. oxycedrus</i>	Shoot apices (2 mm) and nodal segments with 2–3 axillary buds from terminal shoots from 30-year-old trees	Modified SH without PGRs, 3% sucrose, 0.7% agar, pH 5.8. 60 d (SIM) Modified SH + 0.11 mg L <sup>-1</sup> BA (SMM) Modified SH + 0–4.66 mg L <sup>-1</sup> NAA, 0–4.38 mg L <sup>-1</sup> IAA or 0–5.08 mg L <sup>-1</sup> IBA or auxin combinations 30 d. Subculture to the same medium without PGRs. 30 d. (RIM)	26 ± 2 °C, 16-h PP, FTL 80 μmol m <sup>-2</sup> ·s <sup>-1</sup>	Gomez and Segura (1995a) [36]
<i>J. oxycedrus</i>	Leaves (0.5 cm) from shoots growing in vitro obtained from terminal shoots of 30-year-old wild trees	SH + 0.11 mg L <sup>-1</sup> BA, 3% sucrose, 0.7% agar, pH 5.8. 45 d. (SIM)	26 ± 2 °C, 16-h PP, FTL 80 μmol m <sup>-2</sup> ·s <sup>-1</sup>	Gomez and Segura (1995b) [53]
<i>J. oxycedrus</i>	Two studies: 1) Leaves (0.5 cm) from shoots growing in vitro obtained from terminal shoots of 30-year-old wild trees 2) Calli derived from single cells from leaf-derived calli cultured in vitro	1) Modified SH + 1.33 mg L <sup>-1</sup> 2,4-D. 45 d. (CIM) Modified SH + 0.01–1.33 mg L <sup>-1</sup> 2,4-D + 0.01–2.25 mg L <sup>-1</sup> BA. 45 d. (SIM) Modified SH + 1.33 mg L <sup>-1</sup> 2,4-D or 2.41 mg L <sup>-1</sup> picloram. 90 d. (EIM) 3% sucrose, 0.7% agar, pH 5.8. 2) Modified SH + 0.11 mg L <sup>-1</sup> NAA + 1.12 mg L <sup>-1</sup> BA + 100 mg L <sup>-1</sup> CH. 60 d. (SIM) Modified SH + 0.01–0.11 mg L <sup>-1</sup> NAA + 0.11–0.22 mg L <sup>-1</sup> KIN or 0.22 mg L <sup>-1</sup> Zea + 100 mg L <sup>-1</sup> CH. 60 d. (EIM)	26 ± 2 °C, 16-h PP, FTL 80 μmol m <sup>-2</sup> ·s <sup>-1</sup>	Gomez and Segura (1996) [54]
<i>J. cedrus</i>	Mature zygotic seeds	$\frac{1}{2}$ QP + 1.12 mg L <sup>-1</sup> BA, 3% sucrose, 0.8% agar. 15 d. (SIM) Subculture to the same media without BA. 2 m. (SDM) $\frac{1}{2}$ QP or $\frac{1}{2}$ SH + 0.05% AC, 2% sucrose. 6–7 m. (SEM)	24 ± 1 °C, 16-h PP, 60–80 μmol m <sup>-2</sup> ·s <sup>-1</sup>	Harry et al. (1995) [17]
<i>J. excelsa</i> (synonym <i>J. procera</i> )	Two studies: 1) Cotyledons from 5–9-day-old germinating seedlings 2) Zygotic embryos (4–9 mm)	1) Eriksson + 0.5 mg L <sup>-1</sup> BA + 0.02 mg L <sup>-1</sup> NAA. 6w. (SIM) 2) MS + 1.0 mg L <sup>-1</sup> BA + 0.02 mg L <sup>-1</sup> NAA. 12 w. (SIM) 3% sucrose, 0.7% agar, pH 5.6.	25/18 °C (day/night), 16-h PP, 85 μE m <sup>-2</sup> ·s <sup>-1</sup> , RH 86%–92%	Negussie (1997) [18]
<i>J. chinensis</i>	Leaves of young trees	SH + 3.0 mg L <sup>-1</sup> NAA + 0.2 mg L <sup>-1</sup> KIN. 20 d. (CIM). SH + 3.0 mg L <sup>-1</sup> NAA + 0.2 mg L <sup>-1</sup> KIN. 39 d. (CMM) 3% sucrose, 1% agar, pH 5.8.	25 °C, darkness	Muranaka et al. (1998) [13]
<i>J. excelsa</i>	Shoots with apical or lateral buds and needles from 8-year-old plants	Modified MS ( $\frac{1}{2}$ NH <sub>4</sub> NO <sub>3</sub> + $\frac{1}{2}$ KNO <sub>3</sub> ) + Glutamine 100 mg L <sup>-1</sup> + 0.1 mg L <sup>-1</sup> BAP + 0.5 mg L <sup>-1</sup> 2,4-D (CIM) Modified MS (without NH <sub>4</sub> NO <sub>3</sub> ) (SIM)	25 °C, light, 16-h PP	Shanjani (2003) [40]

Table 1. Cont.

Species	Explant Type, Size and Origin	Culture Medium, PGRs and Activities	Culture Conditions	References
<i>J. phoenicea</i>	Microcuttings with axillary buds (1 cm) from cuttings from terminal branches of 20-year-old trees	DKW + 0.20 mg L <sup>-1</sup> KIN or 0.10 mg L <sup>-1</sup> BA or OM + 0.51 mg L <sup>-1</sup> NAA + 0.40 mg L <sup>-1</sup> KIN (SIM) DKW + 0.51 mg L <sup>-1</sup> NAA + 0.40 mg L <sup>-1</sup> KIN or OM + 0.10 mg L <sup>-1</sup> BA or 0.51 mg L <sup>-1</sup> NAA + 0.40 mg L <sup>-1</sup> KIN (SEM) OM + 0.49 mg L <sup>-1</sup> IBA (5 min) (RIM)	22 ± 1 °C, 16-h PP, 98 μmol m <sup>-2</sup> ·s <sup>-1</sup>	Loureiro et al. (2007) [2]
<i>J. procera</i>	Immature dominant and non-dominant zygotic embryos	$\frac{1}{2}$ LP + 1.99 mg L <sup>-1</sup> 2,4-D + 0.10 mg L <sup>-1</sup> BA, 0.4% gellan gum, pH 5.8 (EIM) $\frac{1}{2}$ LP suspension culture (CMM) $\frac{1}{2}$ LP + 16–256 mg L <sup>-1</sup> ABA + 7.5% PEG 4000, pH 5.8. (EMM)	22 ± 2 °C, darkness	Belaineh (2009) [50]
<i>J. communis</i>	Megagametophytes with zygotic embryos in the phase of cleavage polyembryony	$\frac{1}{2}$ LP without PGR 4 w. (EIM) 1/6 medium without PGR. 3 m. (EPM) Medium 923 with decreased content of N and Ca without ABA. 3 w. Then, + 15.86 mg L <sup>-1</sup> ABA (EMM) Mature embryos partially desiccated. 3–4 w. Transfer to $\frac{1}{4}$ DCR 4 w. (RIM)	Darkness, 22 °C (embryogenic cell line induction); 120 μmol m <sup>-2</sup> ·s <sup>-1</sup> , 22 ± 1 °C (somatic embryo maturation)	Helmersson and von Arnold (2009) [5]
<i>J. communis</i>	Spring buds from shoots of female and male trees	MS + 0.1 mg L <sup>-1</sup> BA + 4.0 mg L <sup>-1</sup> IBA or 1 mg L <sup>-1</sup> 2,4-D, 3% sucrose, 0.7% agar, pH 5.7. 2 m. (CIM) MS + 2 mg L <sup>-1</sup> BA + 1 mg L <sup>-1</sup> 2,4-D. 1 m. (SIM) MS + 2.0 mg L <sup>-1</sup> BA. 1 m. (SEM)	25 ± 2 °C, 16-h PP, 330 μmol m <sup>-2</sup> ·s <sup>-1</sup>	Kocer et al. (2011) [23]
<i>J. navicularis</i>	Shoot tips and nodal segments of young shoots originating from mature male and female plants	GD + 0.20 mg L <sup>-1</sup> BA + 0.51 mg L <sup>-1</sup> NAA, 2% sucrose, 0.8% agar, pH 5.8. 1 m. (SIM) GD + 0.10 mg L <sup>-1</sup> BA. 1 m. (SMM) OM + 2.5 mg L <sup>-1</sup> IBA without l-glutamine. 6 w. (RIM)	16-h PP, 24/19 °C day/night, 90 μmol m <sup>-2</sup> ·s <sup>-1</sup>	Castro et al. (2011) [4]
<i>J. excelsa</i>	Young shoot tips from trees	WPM + 0.5 mg L <sup>-1</sup> BA + 0.5 mg L <sup>-1</sup> 2,4-D + 5% sucrose, pH 5.7. 12 w. (CIM) WPM + 0.5 mg L <sup>-1</sup> BA + 5% sucrose, pH 5.7. 16 w. (SDM, SEM) WPM + 0.1 mg L <sup>-1</sup> IBA + 5% sucrose, pH 5.7. 4–6 w. (RIM)	25 ± 2 °C, WIT, 16-h PP	Zaidi et al. (2012) [3]
<i>J. horizontalis</i>	Young shoot tips from trees	WPM + 0.5 mg L <sup>-1</sup> BA + 0.5 mg L <sup>-1</sup> 2,4-D + 5% sucrose, pH 5.7. 12 w. (CIM) WPM + 0.5 mg L <sup>-1</sup> BA + 5% sucrose, pH 5.7. 16 w. (SDM, SEM) WPM + 0.1 mg L <sup>-1</sup> IBA + 5% sucrose, pH 5.7. 4–6 w. (RIM)	25 ± 2 °C, WIT, 16-h PP	Zaidi et al. (2012) [3]
<i>J. chinensis</i>	Young shoot tips from trees	WPM + 0.5 mg L <sup>-1</sup> BA + 0.5 mg L <sup>-1</sup> 2,4-D + 5% sucrose, pH 5.7. 12 w. (CIM) WPM + 0.5 mg L <sup>-1</sup> BA + 5% sucrose, pH 5.7. 16 w. (SDM, SEM) WPM + 0.1 mg L <sup>-1</sup> IBA + 5% sucrose, pH 5.7. 4–6 w. (RIM)	25 ± 2 °C, WIT, 16-h PP	Zaidi et al. (2012) [3]

**Table 1.** Cont.

Species	Explant Type, Size and Origin	Culture Medium, PGRs and Activities	Culture Conditions	References
<i>J. phoenicea</i>	Microcuttings from 3-year-old seedlings	OM + 0.5 mg L <sup>-1</sup> TDZ. 3 w. (SIM, SDM) OM + 0.5 mg L <sup>-1</sup> TDZ. 2 m. (SEM)	25 ± 2 °C, 16-h PP, 40 W, FTL	Al-Ramamneh et al. (2012) [7]
<i>J. excelsa</i>	Lateral bud cuttings from 8-year-old plants	MS + 3.0 mg L <sup>-1</sup> 2,4-D + 0.2 mg L <sup>-1</sup> KIN. 3% sucrose, 0.7% agar, pH 5.7–5.8. 30 d. (CIM)	25 ± 2 °C, darkness	Baravardi et al. (2014) [21]
<i>J. virginiana</i>	Fresh leaves from mature trees	SH + 3.0 mg L <sup>-1</sup> NAA + 0.2 mg L <sup>-1</sup> KIN. 4 w. (CIM) SH + 3.0 mg L <sup>-1</sup> NAA + 0.2 mg L <sup>-1</sup> KIN + 15 mg L <sup>-1</sup> AA. 25 d. (CPM)	25 ± 2 °C, 16-h PP, FTL 3500 lux	Kašparová et al. (2016) [57]
<i>J. phoenicea</i>	Microcuttings (0.5 and 1.5 cm) from branches of 3-year-old seedlings	OM + 0.1 mg L <sup>-1</sup> TIBA. 1 m, 2 m, 3 m. (SIM, SEM, RIM)	No information	Al-Ramamneh et al. (2017) [19]
<i>J. polycarpos</i>	Shoot tips with 1–3 lateral buds from about 7-year-old trees	OM + 0–10 mg L <sup>-1</sup> KIN or BA, 5% sucrose, pH 5.8. (SIM) OM + 5 mg L <sup>-1</sup> KIN or BA, 5% sucrose, pH 5.8. (SEM)	25 °C, 16-h PP, WFT, 5000 lux	Momeni et al. (2018) [9]
<i>J. excelsa</i>	Young shoot tips (1–1.5 cm)	WPM + 0.5 mg L <sup>-1</sup> BAP + 0.5 mg L <sup>-1</sup> 2,4-D. 9 w. (CIM, SIM) WPM + 0.1 mg L <sup>-1</sup> IBA. 6 w. (RIM)	No information	Kashani et al. (2018) [15]
<i>J. thulifera</i>	Shoots with at least one axillary bud from 8–10-year-old trees (1.0–1.5 cm)	WPM + 0.5 mg L <sup>-1</sup> BAP + 1 mg L <sup>-1</sup> 2,4-D, vitamins, 3% sucrose, 0.7% agar, 0.25 g L <sup>-1</sup> AC. 30–45 d. (CIM) WPM + 0.5 mg L <sup>-1</sup> BAP + 0.25 mg L <sup>-1</sup> 2,4-D. 4 w. (SDM) WPM + 0.5 mg L <sup>-1</sup> BAP + 0.25 or 1 mg L <sup>-1</sup> 2,4-D (SEM) $\frac{1}{2}$ MS + 5 mg L <sup>-1</sup> IBA + 5 mg L <sup>-1</sup> NAA, 1.0 g L <sup>-1</sup> AC. 5–7 w. (RIM)	25 ± 2 °C, darkness (callus induction); 22 ± 2 °C, 16-h PP, 1500 lx 'white nothingness' (shoot multiplication); 22 °C, darkness, 16-h PP (shoot rooting)	Khater and Benbouza (2018) [22]

AA—ascorbic acid; ABA—abscisic acid; AC—activated charcoal; BA—6-benzyladenine; CH—casein hydrolysate; CIM—callus induction medium; CMM—callus maintained medium; CPM—callus proliferation medium; CW—coconut water; CWFL—cool-white fluorescent lamp; 2,4-D—2,4-dichlorophenoxyacetic acid; DKW—Driver and Kuniyuki (1984) [58]; EIM—somatic embryogenesis induction medium; EMM—somatic embryo maturation medium; EPM—embryogenic tissue proliferation medium; FTL—fluorescent tube lights; GD = DCR—Gupta and Durzan (1985) medium; IAA—indole-3-acetic acid; IBA—indole-3-butyric acid; IM—induction medium; KIN—N<sub>6</sub>-furfuryladenine; LP—von Arnold and Clapham (2008) medium; MM—maintenance medium; MS—Murashige and Skoog (1962) medium; NAA—1-naphthaleneacetic acid; SDM—shoot development medium; SEM—shoot elongation medium; SIM—shoot induction medium; SMM—shoot multiplication medium; OM—Rugini olive (1984) medium [59]; PEG—polyethylene glycol; PGR—plant growth regulators; picloram—4-amino-3,5,6 trichloropicolinic acid; PP—photoperiod; QP—Quoirin and LePoivre (1977) medium; RH—relative humidity; RIM—root induction medium; SH—Schenk and Hilderbrandt (1972) medium; TDZ—thidiazuron; TIBA—2,3,5-triiodobenzoic acid; WIT—white inflorescent tube lights; WFT—white fluorescent tubes; WPM—McCown Woody plant (1981) medium [60]; Zea—Zea mays.

Recently, it was shown that the induction of callus in junipers is correlated not only with plant growth regulator (PGR) combinations, but also with other factors, such as genotype, sampling time and even gender [23]. The authors obtained good quality calluses for *J. communis* from spring buds explants (up to 90% induction frequency) capable of regenerating numerous adventitious buds that developed in shoots. Moreover, halving the concentration of nitrogen in the MS medium and supplementation with glutamine resulted in a stimulation of callus production for *J. excelsa* [49]. The obtained calluses were able to regenerate the adventitious buds.

In conclusion, during the establishment of juniper callus cultures, attention should be paid both to the age and origin of the explants and to the type and composition of the media used. For the long-term

maintenance of the obtained calluses, suspension cultures seem to be much more effective than agar-solidified media for some species. However, there is a need to more completely investigate the physiological state of the proliferated juniper callus under such conditions. To stimulate organogenesis from calluses, the auxin/cytokinin balance and nitrogen sources in the proliferation media should be much more widely tested.

#### 4.3. Axillary Shoot Multiplication

Micropropagation by axillary shoots is the very effective method for the large-scale propagation of numerous plant species. The culture is established from shoot apices or fragments of shoots with an apical meristem or axillary bud. According to the literature, positive results from this method have also been reported for many hardwood species [61]. Although it presents more difficulties in conifers [62], it is a simple and promising method for the in vitro propagation of *Juniperus* species [2–4,22,36,55]. The successful application of this method was first reported for *J. oxycedrus* [36]. The authors obtained 5.2 shoots per explant using shoot apices and nodal segments, which rooted at 7%–10% frequency (Table 1). Microcuttings with axillary buds [2,4,7,19,22] or shoot tips [3,9,15,22] were the most often used explants for establishment of axillary shoot cultures. The efficiency of induction of shoots from these types of explants varied among *Juniperus* species from 4 to 12.9 per explant for *J. navicularis* and *J. thulifera*, respectively [4,22]. It was revealed that the type of medium applied determined the number of shoots per explant, the shoot length and the number of branches per shoot in *J. phoenicea* [2]. Similar results were reported for *J. navicularis* and for *J. phoenicea* [4,7]. Some studies showed that the proliferation response of some juniper species increased during subsequent subcultures [2–4]. This resulted from the reduction in the level of stress that occurs after the first adaptation period to the in vitro conditions [2].

Successful growth of the axillary shoots of various juniper species was obtained on different media, such as SH [36], OM [2,7,9,19], WPM [3,15,22], GD = DCR [4,48] and DKW [2]. It was demonstrated that the content of total nitrogen in the media may affect the morphological responses of the cultured explants [9]. The highest number of shoots per explant in *J. polycarpos* was produced on the OM medium [59], which was lower in nitrogen content than the MS medium. Similar results were reported for *J. phoenicea*. Such a relationship was found between the OM and MS media composition and the number of shoots per explant [7]. For *J. excelsa*, evaluation of the effects of various media components was made in order to optimize the protocol for in vitro micropropagation [15]. However, of the analyzed components, L-glutamine, which is an important source of organic nitrogen in the medium, did not affect the regeneration of axillary buds or shoots. In turn, the multiplication rate of juniper shoots may be affected by the concentration of the gelling agent added to the medium. It was demonstrated that a higher concentration of gellan gum ( $6\text{ g L}^{-1}$ ) slightly reduced the multiplication and the abnormal appearance of *J. navicularis* buds during the second cycle of proliferation [4].

Plant growth regulators play a key role in the micropropagation of woody plants. Shoot multiplication of *J. oxycedrus* was dependent on the type and concentration of cytokinins applied to the proliferation medium [36]. The highest multiplication rate was obtained after application of  $0.11\text{ mg L}^{-1}$  BA to the SH medium, and the greatest shoot length was noted when the medium was additionally supplemented with  $0.01\text{ mg L}^{-1}$  KIN. For *J. navicularis*, BA at  $0.1\text{ mg L}^{-1}$  applied to the OM or GD medium was the best PGR and obtained the most efficient multiplication rate [4]. The concentration of BA at  $0.5\text{ mg L}^{-1}$  in WPM was the best for the proliferation of three juniper species (*J. excelsa*, *J. horizontalis* and *J. chinensis*) shoots [3]. Recently, it was demonstrated that the separate use of BA and KIN had a significant effect on the number of shoots per explant and shoot elongation in *J. polycarpos* [9]. Moreover, the shoot multiplication rate and the shoot length increased with increasing KIN or BA concentration from 1 to  $5\text{ mg L}^{-1}$ . The authors concluded that the stimulation of shoot production and the inhibition of shoot length could be related to the high activity of these cytokinins. To improve caulogenesis in conifers, pulse treatment has often been used [63,64]. However, attempts undertaken did not have positive results because of a progressive loss of morphogenetic potential and

an increase in the hyperhydricity of the explants after their exposure to the liquid medium containing  $11.26 \text{ mg L}^{-1}$  BA [36].

Efficient axillary bud differentiation and the highest shoot multiplication rate were reported for *J. phoenicea* [7]. The authors applied another type of cytokinin, TDZ, at  $0.5 \text{ mg L}^{-1}$  to OM medium. Shoots and secondary branches developed on this medium, and PGR combinations were thicker, more swollen and darker green than those developed with KIN. However, after two months of exposure to TDZ, the shoots started to callus. The highest elongation rates and the optimal number of shoots per explant were observed, as well as the branches per shoot when both auxin NAA ( $0.51 \text{ mg L}^{-1}$ ) and cytokinin KIN ( $0.40 \text{ mg L}^{-1}$ ) were applied to the DKW medium [2]. In *J. thurifera*, the best results were observed on WPM medium supplemented with  $0.25 \text{ mg L}^{-1}$  BA and  $0.25 \text{ mg L}^{-1}$  2,4-D [22]. Higher PGR concentrations promoted shoot elongation in the first subculture, but lower concentrations were required in the following subcultures. According to previous reports, Cupressaceae respond favorably to relatively low rates of cytokinins in the medium [18], which is confirmed by most of the cited research.

#### 4.4. Adventitious Shoot Multiplication

In conifers, multiplication via adventitious shoots is less effective than the axillary shoot proliferation method. Adventitious buds regenerate from previously-formed meristematic centers that appear in explants or calluses as a result of stimulation with specific PGRs. However, for most coniferous species, the number of shoots produced by adventitious budding is not satisfactory. Despite this fact, attempts to micropropagate junipers using this method have been made for several species (Table 1).

Callus and adventitious buds were induced from various explant types, including leaves from shoots growing in vitro [16], mature zygotic seeds [17], cotyledons from germinating seedlings [18], shoots with apical or lateral buds and needles [49], spring buds [23] or microcuttings from branches of 3-year-old seedlings [19]. Adventitious shoots of *J. oxycedrus* were also obtained from single-cell-derived calluses [54]. The induction of this type of bud has been reported on MS or modified MS, SH,  $\frac{1}{2}$  QP, half strength Quoirin and LePoivre (1977) [65], OM and Eriksson and Eriksson (1965) media [16–19,23,49,53,66]. Adventitious buds of *J. communis* were induced from spring buds on full-strength MS medium supplemented with various concentrations of BA and 2,4-D [23]. However, the further formation of adventitious buds was stimulated by high cytokinin concentration and lack of auxin in the medium. To promote shoot growth, auxin was also excluded from the shoot elongation medium. For comparison, better bud regeneration from leaf explants of *J. oxycedrus* was obtained when MS macronutrients were diluted to one-half strength and the medium was supplemented with  $0.11 \text{ mg L}^{-1}$  BA [53]. In turn, satisfactory results were obtained after the elimination of ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) from the MS medium and the possible supplementation with glutamine [49]. Previous studies on the influence of media components on embryonic explants of *Pinus strobus* revealed that high ammonium content in full-strength MS medium prevented shoot formation [67]. The proper balance between ammonium and nitrate should be maintained to obtain adventitious shoot formation in conifers [68]. The percentage of caulogenesis and the number of buds per explant depended on the macronutrients (especially on the ratios of ammonium, nitrate and potassium) in the media when leaves from in vitro cultures of *J. oxycedrus* were used as explants [53]. The authors reported that SH medium, which is lower in nutrients than MS medium, was the most favorable for the expression of caulogenic responses in this juniper [16,53,54]. Bud regeneration occurred both without PGRs and in the presence of  $0.11 \text{ mg L}^{-1}$  BA in this medium. The bud-forming capacity was also dependent on the cytokinin concentration used (BA or KIN), and was reduced in the presence of NAA in the medium [16]. Moreover, the type of culture significantly determined the success of micropropagation by adventitious buds. Agar culture was more suitable for bud regeneration than liquid culture, in which a high degree of hyperhydricity was observed after an incubation time longer than 21 days [16]. In the case of *J. cedrus*, explants responded well to culture on  $\frac{1}{2}$  QP medium with the addition of  $1.12 \text{ mg L}^{-1}$

BA [17]. However, further bud development required the elimination of BA from the proliferation medium. Shoot elongation was obtained on two media,  $\frac{1}{2}$  QP and  $\frac{1}{2}$  SH, in the presence of 0.05% activated charcoal. In turn, for *J. excelsa* it was noted that 90% of cotyledon and 100% of zygotic embryo explants produced shoots on Eriksson and MS media, respectively [18]. BA at 1 mg L<sup>-1</sup> without or with 0.02 mg L<sup>-1</sup> NAA was indispensable for the initiation of active morphogenic responses and the further growth of adventitious shoots in this species. Similar to *J. cedrus*, shoots of *J. excelsa* grew very quickly on the medium without PGR. Recently, sporadic adventitious shoot induction was reported for *J. phoenicea* [19]. The authors applied OM medium supplemented with daminozide (DM) or TIBA at 0.1 mg L<sup>-1</sup>. However, this medium-PGR combination promoted axillary, rather than adventitious, shoot differentiation in the tested juniper species.

Based on the literature regarding *Juniperus* species micropropagation, different effects have been observed after macronutrient modifications and various concentrations and combinations of auxin and cytokinin applications in different media [2,22,23,36]. This means that the regeneration of junipers based on axillary or adventitious bud multiplication is not easy to establish. Therefore, it is still urgent to define specific media and culture conditions for individual species for the successful micropropagation of this group of plants.

#### 4.5. Rooting and Acclimatization

Rooting of the microshoots regenerated through tissue culture in vitro is a very difficult, slow and inefficient process in conifers [55,69]. The lack of success in this process limits the possibilities for the micropropagation of many coniferous species on the commercial scale [4]. NAA, IBA and IAA are auxins most frequently used to obtain successful coniferous shoot rooting both under in vitro and ex vitro conditions.

Other factors affecting rooting are shoot vigor and juvenility, decreasing mineral, sucrose and agar concentrations in the medium and the use of soilless mixes [55]. In junipers, a very high rooting rate (100%) was reported for two species: *J. oxycedrus* and *J. cedrus* [16,17]. Other studies showed that *J. excelsa*, *J. phoenicea*, *J. navicularis* and *J. thurifera* shoots formed roots at rates of 18.3%–60% (Table 1). The shoots of *J. oxycedrus* were successfully rooted under in vitro conditions on modified SH medium with the addition of 0.47 mg L<sup>-1</sup> NAA and 4% sucrose [16]. However, in general, in vitro rooting was successful in most of the tested juniper species when media were supplemented with IBA alone or in combination with NAA [2–4,22]. Recently, attempts at rooting *J. polycarpos* shoots were undertaken, but the obtained results were not satisfactory [9]. This juniper appears to be a difficult species to induce roots in under in vitro conditions in the presence of IBA or NAA. Until now, positive results for acclimatization after in vitro rooting of shoots have been reported for a few junipers: *J. chinensis* (87% survival rate), *J. phoenicea* (70% in “Morphotype I”), *J. horizontalis* (68%), *J. thulifera* (up to 50%), *J. oxycedrus* (50%), *J. excelsa* (42%) and *J. navicularis* [2–4,16,22]. Ex vitro rooting systems are more commonly used in conifers than in vitro rooting systems [54]. A positive effect was reported for *J. cedrus* with the use of a peat:perlite:vermiculite mixture moistened with  $\frac{1}{4}$  QP medium with 1% sucrose and 0.93 mg L<sup>-1</sup> NAA. In this experiment, 80%–100% of shoots were able to develop roots [17]. It was calculated that using this regeneration system, it would be possible to produce over 250,000 plantlets per initial shoot tip explant per year of culture. In turn, in studies undertaken with *J. excelsa*, only 18.3% of shoots rooted in non-sterile commercial compost [18]. However, shoots rooted in compost were harder than those rooted under sterile conditions, which died after being transferred. For comparison, in the studies, where two rooting systems for *J. navicularis* shoots under in vitro and ex vitro conditions were tested, a higher rooting rate (60%) was observed for shoots growing in vitro in the presence of IBA, which was added to the medium as a rooting inducer [4]. For shoots growing in vermiculite after 5 sec treatment of shoots with IBA alone or with a mixture of IBA with AA, the rooting rate was 42.9%. However, the authors highlighted that rooting the shoots of this juniper in substrates was promising due to its positive effect on the later survival of plants in the nursery. These shoots grew faster and were less exposed to different dysfunctions during acclimatization compared to the shoots rooted

in vitro. This was because the shoots rooted in substrates became autotrophic sooner than the shoots rooted under more artificial in vitro conditions [4]. The presented results demonstrate that ex vitro rooting seems to be more efficient for juniper microshoots obtained via micropropagation methods than in vitro rooting. This means that much more attention should be paid to the development of efficient protocols for the successful rooting of obtained plants in natural conditions. Such a solution would be highly desirable from an economic point of view, because it would allow the elimination of acclimatization problems and would significantly reduce the production costs of juniper plants.

#### 4.6. Somatic Embryogenesis

Somatic embryogenesis is a very useful method for the vegetative propagation of plants. It has a potentially high reproduction rate and allows the maintenance of the obtained plant material (embryogenic tissues, somatic embryos) in liquid nitrogen or in the form of artificial seeds. Thus, in the past thirty years, somatic embryogenesis has been one of the most interesting methods for the micropropagation of woody plants, including coniferous species [70–72]. As a very efficient method, it may be used as an alternative multiplication method to the other in vitro techniques. The first attempts to induce this process in junipers were undertaken by Gomez and Segura in 1996 [54]. The leaf explants of mature *J. oxycedrus* trees, which were cultivated on modified SH medium with the addition of 1.33 or 2.21 mg L<sup>-1</sup> 2,4-D or picloram, were used as explants. In most coniferous species, the successful induction of somatic embryogenesis is dependent on the presence of both auxins and cytokinins in the medium [73–75]. However, the results showed that in this juniper species, the induction of a callus was possible only in the presence of auxins. The *J. oxycedrus* callus induction frequency from leaf explants ranged from 6% to 18%. A positive response was also reported after the application of a callus derived from single cells as explants to induce embryogenic callus.

In this case, embryogenic callus was induced in the presence both of auxin (NAA) and of cytokinin (KIN or Zea). The obtained embryogenic cell lines were able to produce somatic embryos, and yet they were not able to develop into plants under the tested culture conditions. A protocol for micropropagation via somatic embryogenesis was also tried to develop for *J. procera* [50]. Zygotic embryos were used as explants, but only non-embryogenic calluses were induced, and no somatic embryos were observed. In contrast, in the same time, very promising results were obtained for *J. communis* [5]. All stages of somatic embryogenesis in this juniper species were documented, starting from embryogenic callus induction with 50% frequency to the germination and development of small emblings (Table 1). The authors proposed the induction of embryogenic cultures from megagametophytes containing zygotic embryos at a very early step of development (the cleavage polyembryogeny phase). This type of explant may be a key type for somatic embryogenesis induction in junipers, especially given that mature zygotic embryos may not respond to the given culture conditions [12]. Moreover, the application of immature zygotic embryos for the multiplication of junipers via somatic embryogenesis was already suggested previously [17]. It is interesting that the induction of the embryogenic callus of *J. communis* from immature embryos did not require the use of PGRs [5]. It was even found that in the presence of auxin (1.99 mg L<sup>-1</sup> 2,4-D) and cytokinin (0.10 mg L<sup>-1</sup> BA), the initiation frequency decreased compared to that in the PGR-free medium, and the proliferation on the medium supplemented with PGRs was very slow. On the other hand, the proliferation of common juniper in the absence of PGRs made it difficult to trigger the switch from proliferation to embryo development. This problem was resolved by the application of a medium with decreased nitrogen and calcium contents and supplementation with 15.86 mg L<sup>-1</sup> ABA. However, the authors reported abnormalities in the germinating embryos, which proliferated new embryogenic tissue from the basal part of the embryo. These abnormalities were the main factor limiting the application of somatic embryogenesis for the micropropagation of *J. communis*. Therefore, many more studies are needed on controlling the transition from the embryogenic to the vegetative stage in this juniper species [5]. An efficient protocol for *J. communis* micropropagation using this method should be developed, especially given that a positive response to cryopreservation was obtained in the

embryogenic cell lines for this juniper species [5]. All thirteen tested lines survived storage in liquid nitrogen ( $-196^{\circ}\text{C}$ ) and showed positive growth after thawing based on the cryopreservation protocol developed for embryogenic cultures of Norway spruce [76]. Therefore, the presented results obtained for *J. communis* indicate that the propagation of *Juniperus* species via somatic embryogenesis may be a promising solution. In combination with the cryopreservation method, it would be an important and efficient biotechnological tool, allowing propagation of these tree species using an in vitro approach. Therefore, much more research should be performed in this field, first for the juniper species, for which the establishment of embryogenic cultures with immature zygotic embryos as explants would be possible.

## 5. Saving Endangered *Juniper* spp. by In Vitro Cultures

Interest in using in vitro techniques to preserve endangered juniper species emerged in the mid-1990s. Until now, the more or less good results were obtained for a few endangered species using various micropropagation techniques [4,5,17,18,22,23]. The method of the induction of adventitious buds and successful rooting of developed shoots proved to be possible for *J. cedrus* and *J. excelsa* [17,18]. For *J. communis*, which is also considered as an endangered species in some areas, induction of adventitious buds via indirect organogenesis, and the embryos development based on somatic embryogenesis, have been obtained [5,23]. However, attempts to develop efficient micropropagation protocols based on these two methods did not allow the obtaining of fully developed plants due to the problem with the root development. This issue still remains to be solved. In turn, positive results were obtained by developing useful micropropagation protocols for two other endangered juniper species: *J. navicularis* and *J. thulifera*, based on micropropagation by axillary shoots [4,22]. In vitro culture protocols developed for these juniper species can be a practical means for their propagation, preservation and conservation.

Results obtained until now suggest that in the near future various micropropagation techniques may be an applicable tool, not only for reproduction of junipers for the commercial purposes, but also for the conservation of endangered species of this group of plants. However, much more investigations are needed to establish more and more effective propagation methods based on in vitro culture.

## 6. Future Prospects

In this mini-review, the current in vitro state of micropropagation of junipers was assessed. Positive results were reported for eight *Juniperus* species. For most of them, micropropagation protocols based on axillary bud multiplication and organogenesis have been developed. In the case of somatic embryogenesis, only one paper reported the conversion of somatic embryos into emblings. There are still problems with rooting in junipers propagated in vitro, irrespective of the method used. Somatic embryogenesis is currently the most promising method for micropropagation in conifers. Thus, much more attention should be paid to using this method for junipers. Embryos at the cleavage polyembryony stage are good explants for inducing embryogenic cultures. In cases when access to full seeds containing immature zygotic embryos is limited (in rare and endangered species, especially), the plant organs originating from in vitro seed germination or other in vitro techniques may be useful sources of explants. The attempted induction of microshoots or somatic embryos from protoplasts isolated from cells originating from in vitro-obtained calluses should also be considered. Moreover, there is a need to develop basic protocols concerning suspension cultures, cryopreservation, and synthetic seed production with respect to *Juniperus* species to deliver an efficient biotechnological production system for these plants using in vitro techniques.

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