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Propagation of Weeping Willow Trees (*Salix babylonica* L.) Using Plant Tissue Culture Technique

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ABSTRACT: This study was conducted in the Plant Tissue and Cell Culture Laboratory, Department of Forestry Sciences, College of Agriculture and Forestry, University of Mosul, during 2024–2025, aimed to establish an optimal in vitro propagation protocol for Weeping Willow (*Salix babylonica* L.) by evaluating four successive stages: initiation, multiplication, rooting, and acclimatization. In the initiation stage, single-node explants were surface-sterilized using commercial bleach (6% sodium hypochlorite) at concentrations of 5, 10, and 15%, combined with immersion times of 5, 10, and 15 minutes, then cultured on hormone-free MS medium. After four weeks, a 15% bleach concentration and a 15-minute immersion time each independently produced the highest rate of contamination-free explants (86%), confirming both factors as key contributors to effective sterilization. In the multiplication stage, single nodes (1 cm long) were cultured on MS medium supplemented with kinetin (Kin) at 0, 1, 2, 3, and 4 mg/L. After eight weeks, 1 mg/L Kin yielded the greatest number of shoots (6.4/node) and leaves (45.4/node), while the hormone-free control produced the greatest shoot elongation (5.4 cm), indicating a trade-off between cytokinin-driven proliferation and shoot length. In the rooting stage, shoots were cultured on half-strength MS medium with indole-3-butyric acid (IBA) at 0.0, 0.5, 1, and 1.5 mg/L; after six weeks, 0.5 mg/L IBA significantly outperformed other treatments, producing the highest root number (19.5/shoot) and root length (6.7 cm). During acclimatization, assessed four weeks after transfer to ex vitro conditions, 85% of auxin-treated seedlings survived regardless of IBA concentration, exceeding survival in untreated controls. Collectively, these findings establish a reliable, reproducible micropropagation protocol for *Salix babylonica* L.—combining 15% sodium hypochlorite for 15 minutes, 1 mg/L Kin, and 0.5 mg/L IBA—supporting efficient large-scale clonal propagation of this species for forestry and landscaping applications.

1. INTRODUCTION

Weeping willow (*Salix babylonica* L.) is an economically and environmentally important tree species belonging to the family Salicaceae, valued for its ornamental use as well as its role in remediating soil contaminated with hazardous substances [6]. The species also holds notable medicinal significance: recent studies have shown that *S. babylonica* possesses potent antimicrobial properties, as [19] reported that methanolic extracts from the leaves and bark of *S. babylonica* contain effective antibacterial agents, while [1, 7] found that compounds isolated from its alcoholic and aqueous extracts could serve as a natural, functional alternative for treating infections caused by *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*—pathogens responsible for intestinal and skin infections as well as food poisoning.

In addition to these medicinal properties, *S. babylonica* is characterized by rapid growth and high biomass accumulation, making it a promising candidate for renewable energy production. Despite these advantages, *S. babylonica* faces significant challenges in propagation using conventional methods, as propagation by seed is limited by the short shelf life and poor storability of the

seeds, while vegetative propagation via cuttings is similarly constrained: only young, semi-woody cuttings taken from small, vigorous tree root successfully, whereas cuttings taken from large trees or from the previous year's growth lose their rooting capacity [4, 9]. Given these limitations, developing an effective in vitro propagation protocol for *S. babylonica* using plant tissue culture is essential, and in recent years, interest in the practical applications of biotechnology—particularly tissue culture—has grown considerably, owing to its reliance on relatively simple methods that do not require complex or costly laboratory equipment [16].

Tissue culture is regarded as one of the most valuable modern biotechnological tools for developing countries, enabling the rapid production of disease-free, high-quality plants, and the technique involves excising a plant part—such as a seed, root, stem, leaf, bud, or pollen grain—and culturing it on a nutrient medium typically containing macronutrients, micronutrients, and a carbon source (most commonly sucrose), with growth regulators often incorporated into the medium as needed, including auxins (IBA, NAA, IAA) to promote root formation and cytokinins (BA, Kin, 2iP) to stimulate shoot growth and callus induction [2, 13].

Several previous studies have examined the micropropagation of *Salix* species using nodal explants; in one study, nodal segments of weeping willow were cultured on MS medium supplemented with BA and Kin individually at concentrations of 0, 0.5, 1, 2.5, and 5 mg/L, as well as in combination with NAA or GA₃ at concentrations of 0, 0.5, 1, and 2.5 mg/L, and one hundred days after culture initiation, cytokinin supplementation (BA and Kin) markedly enhanced proliferation, with the highest number of shoots obtained on medium containing 1 mg/L BA, while the interaction between BA and NAA or GA₃ had no clear effect on proliferation [2]. [14] investigated the micropropagation of weeping willow using nodal explants, testing three concentrations of sodium hypochlorite (1, 2, 3%) across three immersion times (5, 10, 15 minutes) for sterilization, and twenty-one days after culturing, the combination of 2% sodium hypochlorite and 15 minutes of immersion proved most effective, yielding 90% contamination-free explants; during the growth stage, three growth regulators—BAP, NAA, and GA₃—were evaluated, with the best performance in both shoot length (12.4 mm) and shoot number (5.6) achieved at 1 mg/L for each regulator, and for rooting, NAA, IAA, and IBA were each tested at 1 mg/L, with IAA producing the best results, yielding an average of 4 roots per shoot. [12] applied three sterilization treatments to weeping willow nodal explants—1% sodium hypochlorite, 0.1% mercuric chloride, and a dual treatment combining both agents—with the dual treatment achieving the highest sterilization rate, at 71%; for in vitro shoot induction, 2 mg/L benzyladenine (BAP) was applied alone or in combination with 5 mg/L gibberellic acid, 1 mg/L IAA, or 1 mg/L NAA, and BAP applied alone produced the best response, with a 31.6% induction rate and an average shoot length of 6.47 mm. During the multiplication stage, three culture media—Palomo-Ríos, Chung and Carrasco, and MMULT-1—were compared, with the Palomo-Ríos medium yielding the highest multiplication rate (100%) and an average of 6.42 shoots per explant, while for rooting, IBA was tested at 0.25 and 0.5 mg/L; however, the highest rooting rate (92.6%) was obtained on hormone-free medium. In a related species, *Salix myrtilloides* was propagated in vitro from cuttings sterilized using sodium hypochlorite, silver nitrate, or a combination of both, and sodium hypochlorite at 1.5% for 20 minutes produced the best sterilization results; shoots were subsequently cultured on MS medium supplemented with three cytokinins—BA, Kin, and 2iP—at concentrations of 0.1, 0.5, 1, and 2.5 mg/L, with the highest multiplication rates achieved at 0.5 and 1.0 mg/L Kin [17].

Similarly, [8] reported that supplementing MS medium with kinetin at concentrations of 0.5, 1, 2, and 3 mg/L significantly enhanced multiplication in three *Salix viminalis* cultivars propagated from nodal explants, with the best results for both shoot number and total leaf count obtained at 0.5 mg/L Kin.

The aim of study was therefore designed to establish an efficient in vitro propagation protocol for Weeping Willow (*Salix babylonica* L.) using plant tissue culture techniques. Specifically, it aimed to determine the optimal sterilization conditions, growth regulator concentrations, and culture media for each successive stage of micropropagation—initiation, multiplication, rooting, and acclimatization. Ultimately, the study sought to provide a reliable, reproducible protocol to support the large-scale clonal propagation of this economically and environmentally valuable species.

2. MATERIALS AND METHODS

The study was carried out in the Plant Cell and Tissue Culture Laboratory of the Department of Forestry Sciences at the College of Agriculture and Forestry, University of Mosul, from April 1, 2024, to June 1, 2025. The ready-made MS medium recommended by Murashige and Skoog (1962) was used, produced by CAISSON / LABS, USA, California.

2.1 Stage of initiation (sterilizing plant parts and growing them outside the living organism):

The plant parts that were excised were placed under running water, then transferred to a 1-liter container to which common washing liquid (Zahi) purchased from the local market was added for 5 minutes with continuous stirring to remove dust and debris. They were then rinsed by placing them in a strainer under running water for 5 minutes. The plant parts (nodes) (Explanet) excised from weeping willow seedlings were sterilized by soaking them in sodium hypochlorite (NaOCl) solutions at concentrations of 5%, 10%, and 15% (prepared from a commercial stock solution containing 0.6% sodium hypochlorite) for 5, 10, and 15 minutes. Afterward, the plant parts were removed from the sterilizing solution and washed three times consecutively with sterilized distilled water for five minutes each to remove any traces of the sterilizing agent. Then, they were planted in 300 ml glass bottles containing 25 ml of MS medium free of growth regulators, and the results were observed after four weeks of

2.2 Stage of Shoot Multiplication (Nodes)

Sterile plant parts (single nodes) about 1 cm long were used to carry out multiplication experiments. They were planted on full-strength MS medium supplemented with the growth regulator cytokinin, Kinetin (Kin), at different concentrations (1, 2, 3, 4 mg/L), as well as a control treatment without kinetin. Additionally, 30 g/L sucrose and 7 g/L Agar-Agar were added, with two plant parts per bottle. Results were recorded after eight weeks of culture.

2.3. Rooting Stage

The shoots obtained from the direct node multiplication stage were cut to about 1.5 cm and then planted on half-strength MS medium with different concentrations of IBA (0.5, 1, 1.5 mg/L), along with a control treatment without IBA. Results were recorded six weeks after culture.

2.4 Stage of Acclimatizing the Seedlings:

The rooted seedlings from the rooting stage were taken out of the glass bottles they were grown in, and the roots were thoroughly washed with water to remove any traces of the culture medium and attached algae without damaging the roots. They were then planted in plastic pots containing a growing medium made of sand and peat moss in a 1:1 ratio, sterilized the same way as the nutrient media. After planting, the seedlings were watered with distilled water and then covered with transparent white polyethylene covers and kept in the growth room under the same conditions as the initial stage, with watering as needed. Two weeks after transferring the seedlings to the pots, the covers were punctured, and over time, more holes were added and enlarged until the covers were completely removed after four weeks from the start of acclimatization. The covers were then fully removed, and the acclimatized seedlings were transferred to open-field conditions to get used to the outside environment. After eight weeks from the start of acclimatization, the survival percentage was recorded. All plants in the study were grown in the growth room under controlled conditions with a temperature of $24 \pm 2^\circ\text{C}$. Wudu (light intensity of 2000 lux from white fluorescent tubes) with a light cycle of 16 hours of light followed by 8 hours of darkness daily.

All the study experiments were conducted using the Completely Randomized Design (CRD) as either simple or factorial experiments, and the Duncan Multiple Range Test was used to determine the significant differences between treatments [3] using a computer and analyzed with the SAS program (2001) for all experiments at a 5% probability level, with the experiments including 10 replications.

3. RESULTS AND DISCUSSION

3.1. Initiation Stage:

The results in Table (1) shows the results after four weeks of sterilizing the plant parts and planting them on MS medium without growth regulators. It indicates that there are a clear effect of the NaOCl concentration and the soaking duration on the percentage of healthy (contamination-free) nodes. The highest percentage of uncontaminated parts was 86% when soaked in a 15% concentration, and the highest sterilization rate, also 86%, was achieved with a duration of 15 minutes.

As for the interaction between concentrations and soaking times, there was clear significant effect between the treatments. The table shows that the treatments of soaking for 10 minutes at 10% concentration and soaking for 15 minutes at 15% concentration gave the highest sterilization rate, which reached 100%.

The effective impact of the commercial bleach solution containing 0.6% sodium hypochlorite at 10% and 15% concentration, when used to soak plant parts (nodes) for 10 and 15 minutes for sterilization, can be attributed to the fact that this treatment provided the best conditions for eliminating contamination, giving the lowest contamination rate of 0% and the highest survival rate of 100%.

Table 1. Effect of Sodium Hypochlorite (NaOCl) Concentration and Immersion Time on Surface Sterilization of Nodal Explants.

Time period	Concentration NaOCL			Effect of time
	5%	10%	15%	
5	40 c	40 c	70 c	50 b
10	50 bc	100 a	90 b	80 a
15	70 bc	90 b	100 a	86 a
Effect NaOcl	53.3 b	76.6 a	86.6 a	

Means followed by the same letter within each column are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

3.2 Multiplication Stage:

The results in Table (2) show that table also shows no significant differences between the concentrations in the multiplication percentage, as the multiplication percentage was 100% for all concentrations as well as for the control treatment, adding different concentrations of kinetin to the MS medium led to variations in the number of branches produced per node and in the number of leaves. The highest number of branches (6.4 branches/node) was obtained from nodes grown in the medium containing 1.0 mg/L Kin, which was significantly higher than the other concentrations and the control treatment. Regarding the number of leaves, the highest number was observed in nodes grown in the medium with 1.0 mg/L, reaching 45.4 leaves/node, significantly higher than the other concentrations and the control treatment. As for the length of the longest branch, the table shows that the best branch length, 5.4 cm, was obtained from the control treatment, which was significantly higher than the Kin concentrations.

Table 2. Effect of Kinetin (Kin) Concentration on Shoot Multiplication of Single Nodal Explants Eight Weeks After Culture.

Concentration	Characteristics			
	Percentage %	Number of branches	Number of leaves	Length of Longest Branches
0.0	100a	2.5 c	29.4 b	5.4 a
1.0	100 a	6.4 a	45.4 a	4.43 a
2.0	100 a	4.8 b	38.5 ab	2.72 b
3.0	100 a	4.7 b	37.9 ab	2.47 b
4.0	100 a	4.7 b	37.4 ab	2.33 b

Means followed by the same letter within each column are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).



MS 0.0 mg/L Kin 1.0 mg/L Kin 2.0 mg/L Kin 3.0 mg/L Kin 4.0 mg/L

Figure (1): Multiplication of single nodes planted on MS medium prepared with different concentrations of Kin after eight weeks of cultivation.

Cytokinin's play a major role during the multiplication phase by controlling apical dominance and breaking the dormancy of lateral buds in the plant. Cytokinin's work by inhibiting apical dominance, increasing branching, and forming lateral buds, which leads to more branches. The formation of branches and leaves on growing plant parts in the control treatment may be due to their content of nutrients and internally produced hormones in the plant [11]. But its differ by types and concertation by different its types the most type of cytokinin's is kinetin benzyl adenine [17] An increase in the concentration causes a decrease in the number of branches, which could be due to the plant's response to the growth regulator reaching the optimal concentration, and increasing the concentration beyond the optimal level results in reverse effects [20] and this agrees with arrival by [8].

3.3 Rooting stage

The results in Table (3) show that adding IBA to MS medium at half-strength salts led to differences between the IBA concentrations used in terms of root number, longest root length, branch number, longest branch length, and leaf number. The highest number of roots was 19.5 roots/branch at a concentration of 0.5 mg/L, which was significantly higher than the other concentrations and the control treatment. The longest root reached 6.7 cm at the same concentration of 0.5 mg/L, significantly longer than the other concentrations. For the number of branches, the highest number was 4.9 at 0.5 mg/L, and the longest branch reached 9.84 cm at 0.5 mg/L. The maximum number of leaves on the rooted branches was 30.6 at 0.5 mg/L. The table also shows that there were no significant differences between concentrations in rooting percentage, as rooting reached 100% for all concentrations as well as the control treatment.

Table 3. Effect of Different IBA Concentrations on Rooting of Weeping Willow Shoots Regenerated from Nodal Explants Cultured on Half-Strength MS Medium After Six Weeks.

Concentration	Characteristics					Total Number of shoots
	Percentage %	Number of roots	Length of root	Number of Branch	Length of Longest Branch	
0.0	100 a	5.9 c	2.35 c	1.8 b	8.37 ab	19.4 b
0.5	100 a	19.5 a	6.7 a	4.9 a	9.48 a	30.6 a
1.0	100 a	12.1 b	4.37 b	2.8 b	6.35 b	21.2 b
1.5	100a	5.0 c	4.17 b	2.3 b	6.57 b	16.0 b

Means followed by the same letter within each column are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).



Figure (2): Rooting of weeping willow branches on MS medium with half-strength salts prepared with different concentrations of IBA after six weeks of cultivation.

Auxins play an important role in rooting as they are among the most effective substances in forming lateral roots by stimulating cell division in the cambium region and elongating them [4, 18]. They also reduce the time needed for rooting, increase its rate, and stimulate the formation of lateral root primordia by attracting nutrients, which leads to cell division and elongation, resulting in the formation of structural roots in the cutting area [5,10]. IBA is considered one of the most important auxins, as its role lies in the physiological effect of returning specialized parenchyma cells to a meristematic state by losing their differentiation. These cells then divide to form the root primordia, continue to grow and develop into root initials, and then grow out of the stem tissue, forming lateral roots. Adding growth regulators increases the number and length of roots up to the optimal concentration, while [10]. And salt concentration has an effect on root lengths because of the phenomenon of nutrient tropism, which encourages roots to spread far to compensate for the deficiency in nutrients [15].

4. CONCLUSION

This study developed a tissue culture protocol for propagating weeping willow (*Salix babylonica* L.), testing four key stages. For sterilization, 15% bleach for 15 minutes gave the best results (86% contamination-free). For multiplication, 1 mg/L Kinetin produced the most branches (6.4) and leaves (45.4) per node, though branch elongation (5.4 cm) was best without hormones. For rooting, 0.5 mg/L IBA produced the most (19.5) and longest (6.7 cm) roots. For acclimatization, auxin-treated seedlings achieved an 85% survival rate. The study concludes that this protocol offers an effective method for mass-producing disease-free weeping willow seedlings.

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AUTHOR CONTRIBUTIONS

The manuscript was written through contributions of author. author have given approval to the final version of the manuscript.

DECLARATION OF COMPETING INTEREST

The author declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Author: confirm and, if applicable, replace with the required Declaration of Competing Interest statement using the journal's template.

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