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Enhancing salinity tolerance in Salix linearistipularis through advanced plant tissue culture techniques

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Abstract. Salinity is a major environmental challenge that hampers agricultural sustainability by affecting vast expanses of arable land globally, including significant portions of the Songnen Plain, which hosts substantial areas of saline-sodic soil detrimental to most plant life. This study focuses on Salix linearistipularis, a naturally salt-tolerant woody plant endemic to the region, to explore its potential in ecological restoration and soil improvement. Through extensive laboratory experiments, we employed various plant tissue culture methods to ascertain the most effective techniques for regenerating Salix linearistipularis, thereby facilitating its use in combating soil salinization. The research utilized several culture media and hormonal treatments to determine optimal conditions for seed germination, callus induction, and subsequent plant regeneration. Key findings indicate that specific combinations of growth hormones significantly enhance the rate of callus formation and adventitious bud differentiation, essential for the plant's survival and growth in saline conditions. Furthermore, the study demonstrates the potential for these methods to produce genetically stable plants capable of thriving in hostile environments. The findings provide a valuable foundation for future applications in ecological restoration projects aimed at mitigating soil salinity issues.

Keywords: Salix linearistipularis, Plant tissue culture, Saline-sodic soil, Salinity tolerance.

1. Introduction

Soil salinization presents a significant global environmental challenge, impeding the sustainable development of agriculture. Currently, over 20% of arable land and a third of irrigated agricultural land worldwide are adversely affected by salinity issues. Particularly affected is the Songnen Plain, the largest part of the Northeast Plain and one of the three major global concentrations of soda saline soil. This region alone encompasses about 3 million hectares of saline-alkali land, with an annual expansion rate of 1.4%, indicating a rapidly worsening condition. Among the flora in this region, Salix linearistipularis stands out as a notable exception due to its robust salt tolerance and is the only woody plant with significant community distribution in the area. Understanding the molecular mechanisms underlying the salinity tolerance of Salix linearistipularis is crucial for enhancing soil quality and supporting vegetation restoration in these harsh environments [1].

The ecological challenges posed by soil salinization are compounded by unsustainable land use and development practices, leading to a marked decline in the productivity of arable lands and the degradation of grasslands. In response, the use of halophytes like Salix linearistipularis for ecological treatment and soil improvement has garnered increasing attention. These plants not only survive but can

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thrive in environments with soil pH values ranging from 9.0 to 10.5, significantly higher than in most natural soils. Previous studies have documented that the Mongolian willow, a variety of Salix linearistipularis, can adapt well to saline-alkali soils and play a crucial role in ecological balance by potentially lowering the pH levels under its canopy compared to adjacent herbaceous plants.

In tandem with ecological efforts, advances in plant tissue culture have opened new avenues for propagating and enhancing plants like Salix linearistipularis. Plant regeneration, an essential aspect of tissue culture, involves regenerating complete plants from small plant parts, such as through organogenesis and somatic embryogenesis. This approach has seen significant advancements following the discovery of key plant hormones like auxin and cytokinin, which are crucial in directing plant development. By leveraging these tissue culture techniques, it is possible to cultivate plants that are genetically stable and capable of thriving in and rehabilitating saline-affected landscapes. The integration of these methods holds promising potential for restoring degraded soils and improving agricultural productivity in salinized areas.

2. Pathways of Plant Tissue Culture Regeneration in Woody Plants

The field of woody plant tissue culture has seen significant advancements over the decades, particularly in the areas of seedling breeding, seed preservation, and transgenic research. This section explores the four primary pathways of tissue culture regeneration as applied to woody plants like poplars: callus regeneration, organogenesis, protoplast regeneration, and somatic embryogenesis.

2.1. Callus Regeneration Pathway

The callus regeneration pathway is a fundamental method in plant tissue culture. Starting with plant tissues as explants, this process involves dedifferentiation to form calluses, followed by redifferentiation, where the calluses develop plant shoots and eventually mature into complete plants. While this method was the earliest used and remains widely applied, it faces challenges such as lengthy culture times, complex procedural steps, and stringent requirements for sterile environments [2]. Moreover, the callus pathway often fails to maintain stable genetic traits from the parent plant, prompting researchers to explore more efficient alternatives like direct regeneration or protoplast regeneration for certain studies.

2.2. Organogenesis and Protoplast Regeneration

Organogenesis is an advanced regenerative method that involves inducing explants directly to produce adventitious buds, which are then cultured to form complete plants. This method boasts several advantages, such as shorter culture times, simpler procedures, and the preservation of parental genetic traits. Studies, like those by Zhang Qiong et al., have highlighted the genetic stability in plant seedlings obtained through organogenesis, though they have also noted less stability compared to other methods [3].

Conversely, protoplast regeneration involves removing the cell wall from plant cells, termed protoplasts, which then regenerate into new plants. This technique helps overcome the limitations of distant hybridization and enhances plant gene research, providing a broader array of breeding options. Despite these benefits, protoplast regeneration is less commonly reported due to its complex requirements and longer cultivation times, which make it less suitable for many plant species.

2.3. Somatic Embryo Regeneration Pathway

Organogenesis, a sophisticated method in plant tissue culture, is a two-phase process that initially involves the induction of adventitious buds directly from explants. This phase is often mediated by the application of specific cytokinins, such as 6-benzylaminopurine (6-BA) or kinetin, which are known to stimulate the division and growth of cells in the meristematic regions of the tissue. Following bud induction, the second phase involves the elongation and maturation of these buds into shoots under the influence of a lower cytokinin to auxin ratio, which is critical for the transition from cellular proliferation to differentiation [4]. The genetic stability observed in plants regenerated through organogenesis is attributed to the minimal chromosomal rearrangements during the cell division phases. Research by

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Zhang Qiong et al. substantiates this by comparing chromosomal and epigenetic markers between organogenetically derived plants and their parent stock, showing minimal variation which underscores the method's utility in preserving genetic fidelity.

In contrast, protoplast regeneration offers a radical approach to plant regeneration, starting with the isolation of protoplasts—plant cells devoid of cell walls—typically derived from leaf mesophyll or callus tissues. These cells are enzymatically treated to remove the cell wall, creating a protoplasm that can be cultured in media enriched with osmotic stabilizers to prevent the cells from bursting. Regeneration from protoplasts involves several critical stages: first, the osmoticum is gradually reduced to encourage cell wall synthesis; following this, divisions of protoplasts are stimulated using specific hormonal cocktails usually rich in auxins like 2,4-dichlorophenoxyacetic acid (2,4-D), which promote the initial cell division and subsequent callus formation [5]. Protoplast fusion techniques allow for the introduction of genetic material from two different species, facilitating the production of hybrid cells that can potentially grow into hybrid plants, overcoming the natural reproductive barriers. This method is particularly valuable in genetic engineering and biotechnological applications, such as the transfer of resistance traits or new metabolic pathways between incompatible plant species. However, challenges such as high cellular fragility, the need for precise osmotic conditions, and a demanding culture environment render this technique less prevalent compared to other tissue culture methods. The success of plant regeneration in woody species heavily depends on the selection of explants. Various plant parts exhibit different potentials for regeneration, influenced by factors like plant age and tissue type. For example, studies have shown that younger plant tissues generally have higher callus induction rates compared to older tissues. Optimal explant selection is crucial for efficient regeneration, as it affects the formation and growth of plant calluses, ultimately impacting the overall success of tissue culture regeneration [6].

3. Establishment of Leaf Regeneration System for Salix linearistipularis

3.1. Preparation of Culture Media

The foundation of a successful tissue culture experiment is the preparation of the appropriate culture media. From Table 1, each medium is specifically tailored to support the growth and development of Salix linearistipularis under controlled laboratory conditions.

Table 1. Preparation Protocols for Plant Tissue Culture Media

MS Medium Preparation	Begin by sterilizing a 1L Erlenmeyer flask, then add 800mL of distilled water. Dissolve 4.94g of Murashige and Skoog (MS) basal medium and 30g of sucrose. Adjust the pH to 5.8 using KOH and HCl. After ensuring complete dissolution, add 8g of agar, top up with distilled water to reach 1L, autoclave at 120°C for 20 minutes, and store in a 70°C oven until use.
1/2MS Medium Preparation	Clean a 1L Erlenmeyer flask, add 800mL of distilled water, and dissolve 2.47g of half-strength MS medium and 30g of sucrose [7]. Adjust the pH to 5.8 and follow the same procedure as the MS medium for the addition of agar, autoclaving, and storage.
WPM Medium Preparation	In a clean 1L Erlenmeyer flask, combine 800mL of distilled water with 2.41g of Woody Plant Medium (WPM) and 25g of sucrose. Adjust the pH, add agar, autoclave, and store as described previously.

3.2. Preparation of Hormone Stock Solutions

Hormones play a critical role in regulating the growth and differentiation of plant tissues in culture. In Table 2, the preparation of accurate and sterile hormone solutions is crucial for the success of plant regeneration protocols.

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Table 2. Preparation Protocols for Hormonal Solutions and Experimental Controls in Plant Tissue Culture

Auxin Solutions	Indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) solutions are prepared at 1mg/mL by dissolving in minimal ethanol, followed by dilution in distilled water, filtering, and sterilization. Store IBA at -20°C and NAA at -20° C [8].
Cytokinin Solutions	For 6-benzylaminopurine (6-BA) and kinetin (KT), dissolve in a small volume of NaOH (1 mol/L for 6-BA) or HCl (for KT) to achieve a concentration of 1mg/mL. After complete dissolution, dilute, filter, and store at 4°C for 6-BA and -20°C for KT.
Other Hormones	Thidiazuron (TDZ) is prepared at two concentrations, 0.2 mg/mL and 0.002 mg/mL, using NaOH for dissolution. 2,4-dichlorophenoxyacetic acid (2,4-d) is prepared at 5 mg/mL in ethanol. Both are stored at -20°C after filtering and sterilization.
Experimental Control	Zebularine, used as an epigenetic modifier in some plant culture systems, is prepared at 5 mg/mL in distilled water, ensuring thorough dissolution and sterilization before storage at -20°C [9].

3.3. Sterilization and Storage of Reagents

The final step in the preparation of experimental reagents involves the sterilization and proper storage to prevent contamination and ensure the stability of the solutions, as shown in Table 3.

Table 3. Guidelines for Sterilization and Storage of Plant Tissue Culture Media and Hormones

Sterilization Methods	Autoclaving is employed for media containing agar to ensure sterility and the inactivation of any microbial contaminants. Hormone solutions and other sensitive liquids are filtered through a sterile membrane to maintain their integrity and effectiveness.
Storage Conditions	Prepared media are generally stored in a pre-heated oven at 70°C to maintain readiness for immediate use, while hormone solutions are stored at specific temperatures (-20°C or 4°C) based on their chemical nature and stability requirements. This meticulous approach to the preparation, sterilization, and storage of plant culture media and reagents ensures a high success rate in plant tissue culture experiments [10].

4. Methods and Procedures

4.1. Media Preparation and Seed Sterilization

The foundation of our experimental design begins with the preparation of the growth media. First, basic media variants such as 1/2 Murashige and Skoog (1/2MS), full-strength MS, and Woody Plant Medium (WPM) are prepared. The pH is adjusted to 5.8 using KOH and HCl. After setting the desired volume, 8 g/L of plant agar powder is added. The media are then sterilized by autoclaving at 120°C for 20 minutes and subsequently maintained at 70°C to ensure readiness for use. Hormones are added at calculated volumes to the cooled sterile media under aseptic conditions in a laminar flow hood [11]. The germination of Mongolian willow seeds poses unique challenges due to the presence of endophytes and a permeable seed coat. The germination rate of Mongolian willow seeds was statistically calculated in different basic media, the callus induction rate was induced by cotyledon explants in callus induction medium after 14 days, and the germination rate was calculated after differentiation of cotyledon explants in differentiation medium for 25 days.

$$Germination\ rate = \frac{number\ of\ germinated\ seeds}{total\ number\ of\ inoculated\ seeds} \times\ 100\% \quad (1)$$

$$Callus\ induction\ rate\ = \frac{number\ of\ explants\ induced\ from\ callus}{total\ number\ of\ inoculated\ cotyledon\ explants} \times\ 100\% \quad (2)$$

$$Germination\ rate\ = \frac{number\ of\ adventitious\ buds\ induced}{total\ number\ of\ inoculated\ cotyledon\ explants} /\times\ 100\% \quad (3)$$

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The test data were analyzed for ANOVA using SPSS, and the difference significance was detected at the 0.05 level by the Duncan method.

Our improved sterilization method involves the use of pre-autoclaved 1.5mL sterile centrifuge tubes, into which seeds stored at -80°C are placed. The seeds undergo several washing steps with sterile water and dilutions of sodium hypochlorite (NaClO), culminating in multiple rinses to remove residual NaClO. The prepared seeds are then plated on 1/2MS media supplemented with 50 mg/L antibiotic for resistance.

4.2. Callus Induction and Plant Regeneration

Callus induction serves as a pivotal step in plant tissue culture, providing a base from which plants can be regenerated under controlled conditions. In our experiments with Salix linearistipularis, specifically the variety known as Mongolian willow, callus induction begins with the selection of high-quality seedlings that have germinated on optimally prepared media. These seedlings are then used to provide cotyledon explants, which are carefully dissected under sterile conditions to ensure the integrity of the tissue.

The explants are placed on specialized callus induction media, which contains a carefully balanced mixture of hormones designed to encourage callus formation. The primary hormones used include indole-3-butyric acid (IBA) and 6-benzylaminopurine (6-BA), known for their efficacy in promoting cell division and callus initiation [12]. The concentration of these hormones is meticulously optimized; typically, IBA is used at concentrations ranging from 0.5 to 2.0 mg/L, while 6-BA is varied from 0.1 to 1.0 mg/L depending on the desired rate of callus induction and subsequent plant regeneration. Additional treatments may include 2,4-dichlorophenoxyacetic acid (2,4-D) at lower concentrations (0.1 to 0.5 mg/L) to enhance the callus formation rate. The hormonal balance is crucial as it influences not only the rate and quality of callus formation but also the genetic stability of the regenerated plants. This is particularly important for Salix linearistipularis, which is utilized in environmental restoration projects where genetic consistency can impact the overall success of plantings in saline-alkali soils.

Following the initial dark phase, which lasts about two weeks to allow for callus establishment, the explants are transitioned to light conditions to promote photomorphogenesis. This light exposure is carefully controlled to provide optimal wavelengths and intensities conducive to healthy growth and development. The differentiation phase follows callus formation, where the callus tissues are transferred to a differentiation medium containing a modified hormonal composition to promote shoot and root development [13]. This medium typically has a reduced level of auxins and an increased level of cytokinins, which supports the formation of adventitious buds and roots. For instance, the medium might contain naphthalene acetic acid (NAA) and 6-BA in a ratio that favors shoot induction, generally skewed towards higher levels of cytokinins. Throughout these processes, meticulous records are kept of all experimental conditions, including temperature, light intensity, and humidity in the growth chambers, to ensure reproducibility and to identify the optimal conditions for each stage of development. Each batch of explants is monitored daily, and the data collected includes rates of callus induction, success of adventitious bud formation, and eventual rooting percentages.

4.3. Germination and Differentiation Analysis

The experimental outcomes are quantitatively assessed by calculating the germination and callus induction rates. The germination rate is the ratio of seeds that successfully germinate to the total seeds inoculated, expressed as a percentage. Similarly, the callus induction rate is determined by the proportion of explants that form callus after 14 days of culture. The effectiveness of different hormonal treatments in inducing adventitious bud formation and subsequent rooting are also meticulously recorded. Data collected from these experiments are statistically analyzed using ANOVA, with significant differences identified at a 0.05 threshold via the Duncan test. This structured approach allows us to systematically evaluate the impact of various media and hormonal conditions on the growth and development of Mongolian willow [14].

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5. Conclusion

This study successfully demonstrates the potential of Salix linearistipularis, a naturally salt-tolerant species, as a pivotal component in ecological restoration efforts targeted at saline-sodic soils, particularly in the challenging environments of the Songnen Plain. Through detailed experimental procedures encompassing the preparation of various culture media and the application of specific hormonal treatments, we have established optimal conditions for seed germination, callus induction, and plant regeneration of Salix linearistipularis.

Key findings from our research reveal that certain hormonal combinations, particularly those involving IBA, 6-BA, 2,4-D, IAA, and NAA, significantly enhance callus formation and adventitious bud differentiation. These hormonal treatments are instrumental in promoting robust plant growth under saline conditions, thereby contributing to the restoration and improvement of degraded soils. Moreover, our results highlight the efficacy of plant tissue culture techniques in generating genetically stable plants capable of thriving in saline-alkali environments. The successful regeneration and subsequent growth of Salix linearistipularis not only affirm its inherent salinity tolerance but also underscore its potential for large-scale use in ecological restoration projects. The implications of this study for combating soil salinization are profound. By harnessing the capabilities of salt-tolerant species like Salix linearistipularis through advanced tissue culture methods, we can significantly mitigate the adverse effects of soil salinity on agricultural productivity and ecological balance. Future research should focus on refining these tissue culture techniques and exploring the genetic mechanisms underlying salinity tolerance, which will further enhance the practical applications of this valuable species in ecological restoration and sustainable agriculture.

In conclusion, the integration of plant tissue culture and the strategic use of Salix linearistipularis offer promising avenues for addressing the global challenge of soil salinization, ultimately contributing to the restoration of fertile lands and the promotion of environmental sustainability.

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