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Role of Carbon Sources on *in Vitro* Plant Regeneration in Alfalfa (*Medicago sativa* L.)

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Abstract

Carbon sources play a critical role in plant tissue culture. This study evaluates the effects of different carbon sources—including monosaccharide hexoses (fructose, glucose, and galactose), disaccharides (maltose, sucrose, and lactose), and sugar alcohols (mannitol, sorbitol, and glycerol)—at concentrations ranging from 1% - 5% on *in vitro* plant regeneration in alfalfa *(Medicago sativa* L.). All leaf explants successfully induced callus formation when cultured on Gamborg's B5 (B5h) medium supplemented with either monosaccharides or disaccharides in darkness for three weeks. Similarly, somatic embryo induction and maturation were enhanced in media containing monosaccharides (85.4% - 100%) and disaccharides (97.5% - 100%). Among all treatments, maltose at 3% exhibited rapid progression to the globular stage, yielding a 96.9% embryo-to-shoot conversion rate and the lowest incidence of shoot loss. Regenerated plantlets were successfully acclimatized and transferred to soil, achieving survival rates ranging from 50% - 100%, depending on the carbon source. These findings underscore the importance of carbon source selection for optimizing somatic embryogenesis protocols and advancing large-scale micropropagation of alfalfa and related legumes.

Keywords

Carbon Sources, Carbohydrate, Plant Regeneration, *In Vitro*, Alfalfa (*Medicago sativa* L.)

1. Introduction

Alfalfa (*Medicago sativa* L.) is a highly nutritious forage species, widely recognized as the "Queen of Forage" due to its exceptional biomass production, forage qual-

ity, and palatability for ruminants [1]. It ranks as the third-largest commodity crop within the United States, following corn and soybean, reflecting its economic importance in the agriculture and livestock sector. Beyond its primary importance as livestock feed, it also offers significant health benefits for humans as it is rich in essential amino acids, vital vitamins, and a wide array of minerals essential for growth and development [2]. Additionally, it enhances soil fertility by fixing atmospheric nitrogen, hence reducing the inputs of synthetic fertilizers. It is also used as a cover crop in grasslands for efficient weed control, thereby improving land productivity. Furthermore, its potential has been explored in phytoremediation applications [3] and recombinant pharmaceutical proteins [4].

Despite its agronomic and economic significance, improvement in alfalfa through conventional breeding remains challenging due to its autotetraploid genome and a high degree of cross-pollination, which complicates genetic analysis and trait fixation [5]. As a result, biotechnological approaches are increasingly explored to overcome these limitations and enhance its genetic potential. Somatic embryogenesis has emerged as a powerful *in vitro* propagation technique, enabling large-scale production of genetically uniform and disease-free plants from a single somatic cell [6]. Different factors that influence the efficiency of somatic embryogenesis include internal elements, such as genotype and explant type, as well as external influences with abiotic factors (temperature, light, pH, and desiccation treatments) and chemical components (macro- and micronutrients, growth regulators, and carbon sources) [7].

Among these parameters, the selection of optimal carbon source and osmotic agents is vital for *in vitro* plant cells, which require external carbon to maintain osmotic potential for key developmental processes like shoot proliferation, root induction, embryogenesis, and organogenesis [8]. However, not all carbon sources are equally metabolized or effectively utilized by every plant species. Thus, selecting the appropriate carbon source at each developmental stage of somatic embryogenesis is critical to producing healthy, high-quality plantlets without abnormalities [8] [9]. Research from Sul and Korban (1998) [10] demonstrates that the type and concentration of carbohydrates play a crucial role in influencing *in vitro* plant growth in addition to the choice of explant used. Studies have shown that high carbohydrate concentrations can inhibit tissue growth [11]. For instance, Navarro-Alvarez *et al.* (1994) [12] reported that carbohydrate type and concentration influence somatic embryogenesis potential in wheat and other cultures. Verma and Dougall (1977) [13] found that in carrot cultures, alternative carbohydrate sources can support development and embryogenesis as effectively as sucrose.

Although sucrose is the standard carbohydrate source in most tissue culture media, other carbohydrate sources and concentrations have been proven effective for embryogenic callus induction, embryo development, and regeneration [8]. Alternative carbohydrates that support somatic embryogenesis include monosaccharides (glucose, fructose, and galactose), disaccharides (maltose, sucrose, and lactose), and polyols (mannitol, sorbitol, and glycerol), which are sugar alcohols de-

rived from the hydrogenation of carbohydrates [7] [14]-[16]. Studies have demonstrated the effectiveness of fructose in cocoa [8] and stevia [17]; glucose in beech [7] and bitter almond [18]; galactose in spinach [19] and citrus species [15] [20] [21]; lactose in wild carrot [13] and citrus [15]; maltose in apple [22], pea [23], and petunia [24]; mannitol in olives [7] and celery [25]; sorbitol in peach [26] and apple [27]; and glycerol in chicory leaf tissues [28] and citrus species [15] [29].

While numerous studies have examined the influence of different carbohydrates on somatic embryogenesis in various plant species, such as carrots and cacao [8] [16] [30], research specifically on alfalfa remains limited. Existing studies on alfalfa somatic embryogenesis are dated and lack detailed analysis regarding the optimal selection of carbon sources. To address this gap, our present study evaluates the effects of different carbon sources, such as monosaccharide hexoses (fructose, glucose, and galactose), disaccharides (maltose, sucrose, and lactose), and sugar alcohol or polysaccharides (mannitol, sorbitol, and glycerol) at five different concentrations (ranging from 1% - 5%) on alfalfa somatic embryogenesis and plant regeneration techniques. Therefore, the goal is to determine the most effective carbon source to regenerate alfalfa plants effectively and develop a cost-effective plant regeneration protocol.

2. Materials and Methods

2.1. Plant Materials

Alfalfa (*Medicago sativa* L.) cultivar Regen SY was used for this experiment and was obtained from the Western Regional PI Station through the U.S. National Plant Germplasm System. *In vitro* propagation was done using nodal cuttings on 3% sucrose Murashige and Skoog (MS) medium with 0.3% gelrite at 5.7 pH for the regular supply of plant materials. Young, dark green, and fully expanded broad leaves of *in vitro*-grown alfalfa plants were selected as the sample for the study. The selected leaves were scarred and segmented into three pieces of uniform sizes (≈2 mm) with the help of a scalpel and forceps to promote a continuous response across all explants during each phase of the experiment and each treatment, facilitating accurate comparison and observations.

2.2. Plant Regeneration Using Different Carbon Sources

To prepare the callus induction medium, Gamborg B5h medium was used with an adjusted pH of 5.7 ± 0.03 before adding 0.3% gelrite. The medium was then autoclaved at 121˚C for 15 minutes. After cooling, 30 ml/L of stock amino acids, filter-sterilized 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.9 µM kinetin were added into prepared media and poured in 100 mm × 25 mm petri dishes. The medium was individually supplemented with monosaccharide (fructose, glucose, and galactose), disaccharide (sucrose, maltose, and lactose), and sugar alcohols (mannitol, sorbitol, and glycerol) at a wide range of concentrations (1% - 5%) at each stage of somatic embryogenesis. The control in this study consisted of a medium

prepared without the addition of an external carbon source.

Leaf explants of *in vitro*-grown alfalfa plants were subcultured on the prepared medium and incubated in the dark at 24˚C ± 2˚C throughout the three-week callus induction phase. The dark conditions were created by wrapping aluminum foil around the Petri dishes. The experimental treatment was consistent when three-week-old calluses were transferred to Gamborg B5 basal medium with no growth regulators to stimulate embryo formation. The cultures were then maintained in light conditions at 24˚C ± 2˚C for another three weeks (Figure 1).

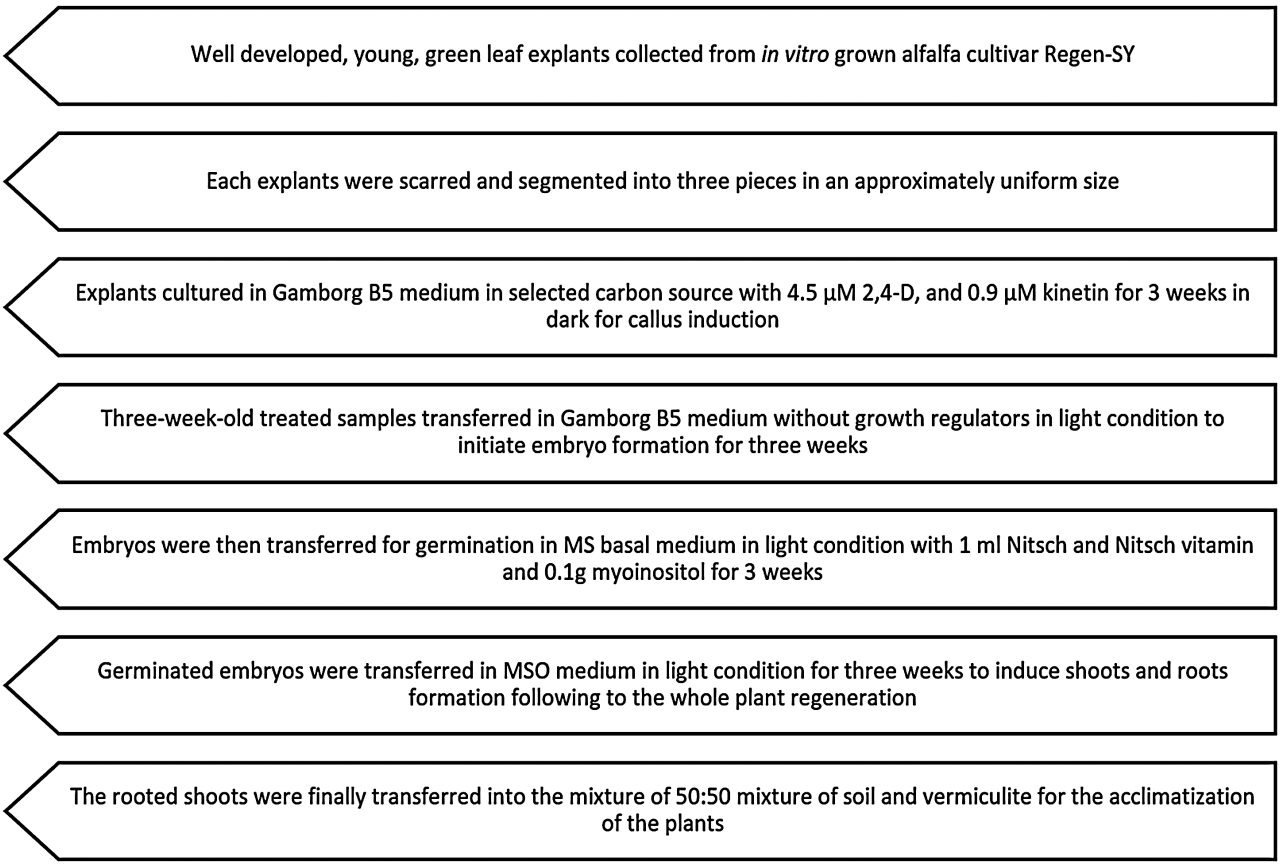

Figure 1. Schematic workflow for plant regeneration in alfalfa (*Medicago sativa* L.).

Once the embryos progressed to the cotyledonary stage, they were transferred into the embryo germination medium in the same light conditions for an additional three weeks. The germination medium consisted of MS basal medium supplied with 1 ml Nitsch and Nitsch vitamin and 0.1 g myoinositol with the pH adjusted to 5.7 ± 0.03, and 0.3% gelrite was added before autoclaving the medium. The different developmental stages of the embryo (globular, heart-shaped, torpedo, and cotyledonary) were monitored using a Leica Microsystems EMSPIRA 3 digital microscope with a magnification of 1.2× (Life Sciences companies, Danaher Corporation, USA). Finally, germinated embryos were transferred into a glass jar containing MS0 medium conditions fostering plantlet formation, still under the influence of the predefined treatment matrix. Regeneration of whole plants was observed

when the plantlets were kept in uniform condition for another three weeks under light conditions. After the plantlets were fully developed, individual plantlet roots were washed with autoclaved distilled water to remove the semisolid culture media. The plantlets were then transferred to sterile 1:1 ratio vermiculite: potting soil mixture and were kept under light for a 16/8-hour photo period, covered with perforated polyethylene bags. The plantlets were supplied with 1/2 strength MS liquid media for 7 days; thereafter, they were watered once weekly.

2.3. Data Collection

Data was taken using the following parameters: callus induction from alfalfa leaf explants, embryo formation from induced callus, embryo conversion, and plant regeneration. Data was recorded after 3 weeks for callus induction, 6 weeks for embryo formation, 9 weeks for embryo conversion, and 12 weeks for plantlets regeneration.

2.4. Statistical Analysis

The study followed a completely randomized design with four replications and was conducted twice. Each treatment included 60 explants, with controls maintained throughout the analysis. Statistical significance was assessed using a two-way Analysis of Variance (ANOVA). Mean values and standard errors were compared using the Least Significant Difference (LSD) test ($p < 0.01$). Bar graphs were created using Prism v. 5.0 (GraphPad Software, La Jolla, CA) and Microsoft Excel, while a heat map was generated using Python 3.11.10 (Python Software Foundation, Wilmington, DE, USA) with the Matplotlib library (version 3.9.2). The response percentage was scored and calculated as follows:

$$\text{Callus Induction } (\%) = \frac{\text{Number of callus induced}}{\text{Total number of explants inoculated}} \times 100$$

$$\text{Embryo Formation}(\%) = \frac{\text{Number of callus forming embryo}}{\text{Total number of induced callus}} \times 100$$

$$\text{Embryo Conversion}(\%) = \frac{\text{Number of convered embryo}}{\text{Total number of mature embryo induced}} \times 100$$

$$\text{Plant Regeneration } (\%) = \frac{\text{Number of regenerated plants}}{\text{Total number of plantlets developed}} \times 100$$

3. Results

This study aims to improve *in vitro* regeneration efficiency in alfalfa by refining the plant regeneration protocol via somatic embryogenesis with an evaluation of nine different carbon sources at varying concentrations as energy supplements. Alfalfa leaf explants were cultured on B5h media containing monosaccharide hexoses (fructose, glucose, and galactose), disaccharides (maltose, sucrose, and lactose), and sugar alcohol or polyols (mannitol, sorbitol, and glycerol) at concentrations ranging from 1% - 5%.

3.1. Effect of Carbon Sources on Embryogenic Callus Induction

During the first week of dark incubation, noticeable tissue swelling occurred in alfalfa explants cultured on media containing monosaccharide hexoses (fructose, glucose, and galactose) or disaccharides (maltose, sucrose, and lactose). In contrast, no such response was observed in alfalfa explants exposed to sugar alcohols or polyols (mannitol, sorbitol, and glycerol). By the second week, substantial callus formation was evident in alfalfa explants treated with monosaccharides and disaccharides. After three weeks, nearly all calli had turned green, with an early indication of somatic embryo formation. As cultivation progressed, the media-supplemented carbon sources supported vigorous callus growth with minimal browning, regardless of their concentration (Figure 2). Conversely, explants cultured in the control group (lacking any carbon source) and sugar alcohols exhibited pale white coloration, irregular and shrunken shapes, curled margins, and no signs of embryogenic callus formation, indicating poor embryonic callus induction.

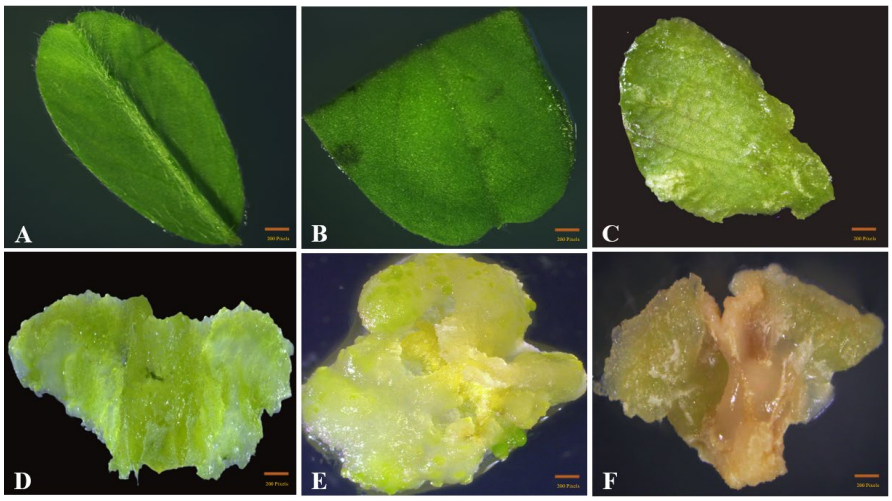

Figure 2. Embryogenic callus induction from *in vitro*-grown alfalfa leaf explants cultivar Regen-SY. **(A)** Fully expanded whole leaf explant (6 mm); **(B)** Excised leaf explants (2 mm) prepared for inoculation in callus induction medium; **(C)** Expansion of leaf tissue after one week of culture; **(D)** Two-week-old embryogenic callus derived from leaf explants; **(E)** Initiation of pro-embryogenic tissue after three weeks of culture; **(F)** Prolonged culture (four weeks) resulting in the conversion of embryogenic callus into non-embryogenic callus, characterized by a loose and watery structure due to the presence of auxin (2,4-D) in the medium.

Although all explants formed callus on media enriched with monosaccharide hexoses and disaccharides, the morphological characteristics of the calli varied significantly depending on the carbon source, such as fructose-enriched media-induced calli with prominent green nodules, indicating active somatic embryo formation (Figure 3(A)). Glucose-containing media yielded compact, dark green calli, suggesting early stages of embryo initiation (Figure 3(B)). Calli that developed on galactose-enriched media were dense and light green, reflecting ongoing embryo

progression (Figure 3(C)). In contrast, maltose-supplemented media generated bright yellow-green calli with well-formed somatic embryo structures (Figure 3(D)). Sucrose-containing media led to the formation of pale yellow calli with little to no organized tissue formation (Figure 3(E)). Calli grown on lactose-supplemented media were composed of parenchymatous cells, implying the initiation of embryogenesis (Figure 3(F)). Minimal and unorganized callus initiation was observed at the leaf margins in media supplemented with sugar alcohols—mannitol (Figure 3(G)), sorbitol (Figure 3(H)), and glycerol (Figure 3(I)).

The quantitative analysis supported these findings, showing that 100% of explants (average number: 12) induced callus in media containing monosaccharide hexoses or disaccharide carbon sources. In contrast, response rates were significantly lower in the control media with only 35.4% explant forming callus followed by mannitol (25% - 33.3%), sorbitol (22.9% - 33.3%), and glycerol (18.7% - 25%) with average number less than 5 in each group as shown in Figure 3 and Figure 4. Alfalfa leaf explants showed limited growth on media supplemented with mannitol, sorbitol, and glycerol, and failed to produce any embryos, highlighting the

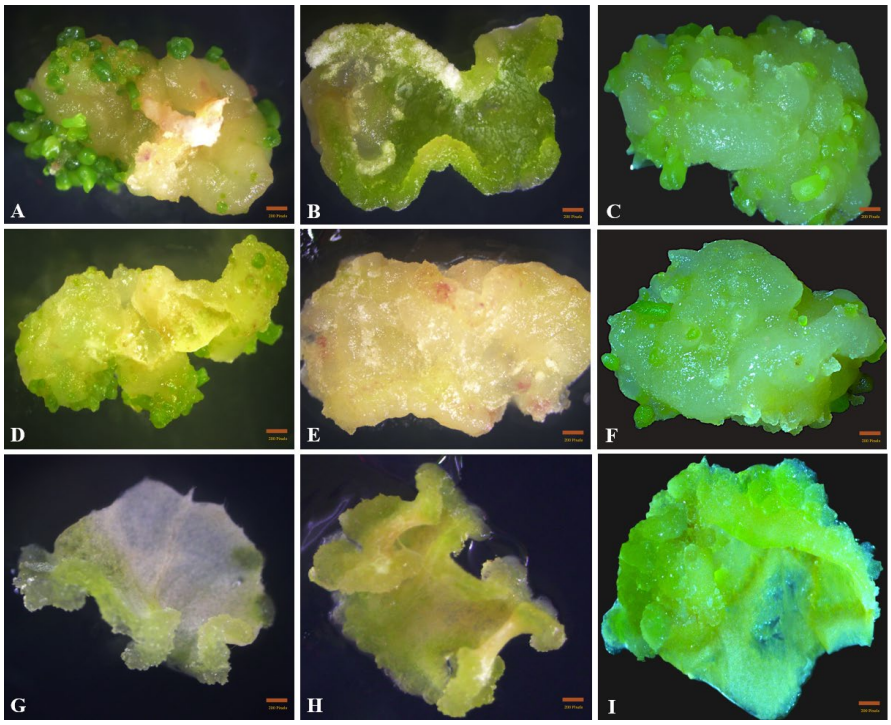

Figure 3. Effect of different carbon sources on callus induction in alfalfa cultivar Regen-SY cultured on Gamborg B5 medium supplemented with individual carbon sources. **(A)** Callus formation on fructose-supplemented medium; **(B)** Callus formation on glucose-supplemented medium; **(C)** Callus formation on galactose-supplemented medium; **(D)** Callus formation with embryo development on maltose-supplemented medium; **(E)** Callus formation on sucrose-supplemented medium; **(F)** Callus formation, predominantly consisting of parenchymatous cells, on lactose-supplemented medium; **(G)** Leaf explant turned pale white on mannitol-supplemented medium; **(H)** Curling and pale white appearance of leaf explant on sorbitol-supplemented medium; **(I)** Folding or shrinking and creamy white appearance of leaf explant on glycerol-supplemented medium.

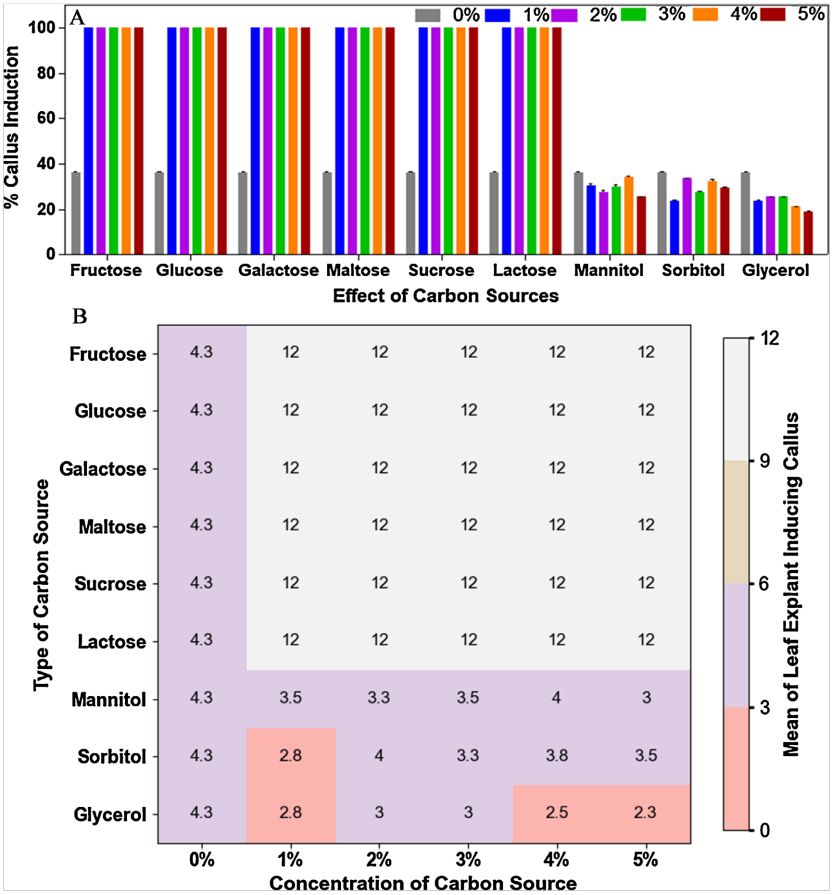

Figure 4. Effect of different carbon sources and their concentrations on callus induction in alfalfa cultivar Regen-SY. **(A)** Percentage of explants inducing callus; **(B)** Mean number of leaf explants inducing callus. Alfalfa leaf explants were cultured as described in **Figure 2** [31], and data were collected three weeks after culture initiation. Each treatment included 60 explants from four replicate plates, with 12 alfalfa leaf explants per plate. Vertical lines indicate the standard error of the means. Significant differences between carbon sources ($p < 0.001$) are indicated with different letters over the bars.

ineffectiveness of these carbon sources in promoting organized callus formation and somatic embryogenesis.

3.2. Effect of Carbon Sources on Embryo Formation and Maturation

After three weeks of embryogenic cell formation, the green areas of the calli were gradually created to exhibit budding. At this stage, the leaf-induced callus was transferred to a B5H hormone-free medium containing respective carbon sources for three weeks and grown under light conditions following the protocol of Filonova *et al.* (2000) [32] to promote embryo maturation and development. The developmental stages of alfalfa somatic embryos progressed sequentially through the globular, heart, torpedo, and cotyledonary phases. The globular stage, representing the early phase of somatic embryogenesis, was characterized by a small, undifferentiated, round-shaped structure. In the heart-shaped stage, the embryo developed a distinct bilobed or heart-like shape, prominently displaying two lobes with a cen-

tral notch. During the torpedo stage, the embryo elongated into a cylindrical form. Finally, the cotyledonary stage was marked by the emergence of one or more cotyledons, indicating further differentiation and development [6].

This study highlighted the effectiveness of somatic embryogenesis by evaluating the number and developmental stages of embryos across different carbon sources. On fructose-supplemented medium, multiple embryos successfully advanced to the torpedo stage (Figure 5(A)). Glucose-enriched medium also supported notable embryo maturation (Figure 5(B)), while galactose primarily led to embryos remaining at the globular stage (Figure 5(C)). The highest levels of embryo formation and maturation were observed in maltose-supplemented medium (Figure 5(D)), likely due to its slow hydrolysis into glucose, providing a consistent energy supply for development. In comparison, sucrose-supported medium resulted in lower rates of embryo maturation (Figure 5(E)), and lactose led to the formation of embryos at the heart stage (Figure 5(F)). Conversely, mannitol (Figure 5(G)), sorbitol (Figure 5(H)), and glycerol (Figure 5(I)) failed to support any noticeable embryo formation.

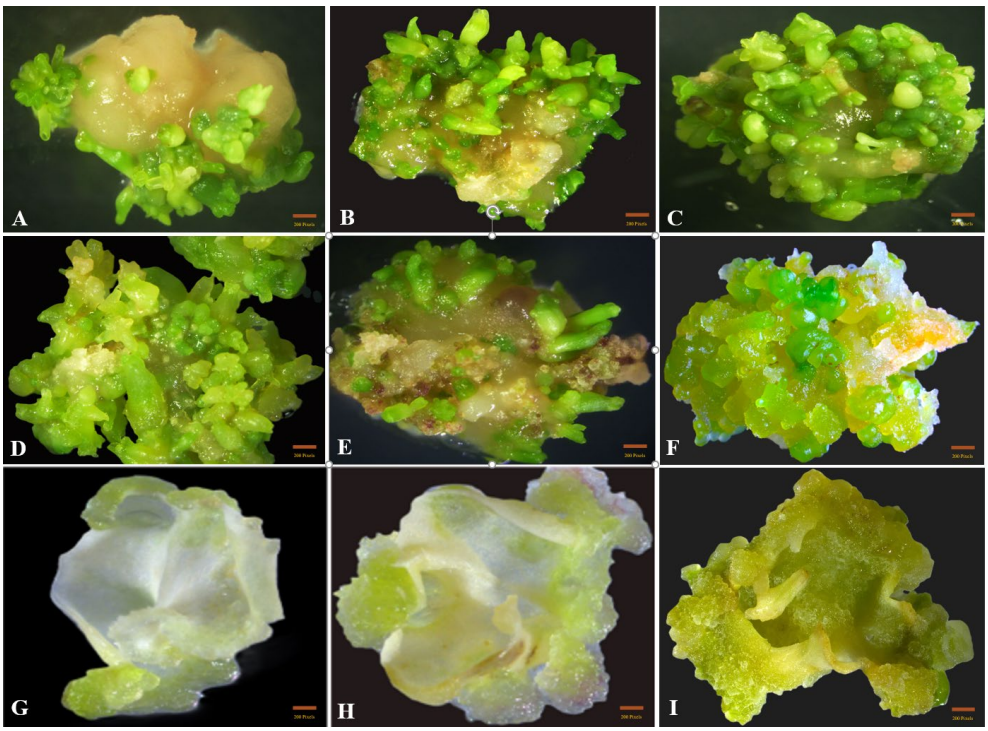

Figure 5. Effect of different carbon sources on callus formation and somatic embryo maturation in alfalfa cultivar Regen-SY cultured with specific carbohydrates at a 3% concentration on Gamborg B5H hormone-free medium. **(A)** Embryo development observed on fructose-supplemented medium, with some areas of callus remaining untransformed; **(B)** Embryo development on glucose-supplemented medium; **(C)** Significant embryo development on galactose-supplemented medium; **(D)** Dense and highly expressed mature embryos observed on maltose-supplemented medium; **(E)** Localized embryo formation on sucrose-supplemented medium, with browning in certain areas; **(F)** Moderate embryo growth on lactose-supplemented medium, with some necrotic regions; **(G)** No embryo formation observed on mannitol-supplemented medium; **(H)** Poor embryo formation with partial leaf browning on sorbitol-supplemented medium; **(I)** Formation of anembryonic tissue observed on glycerol-supplemented medium.

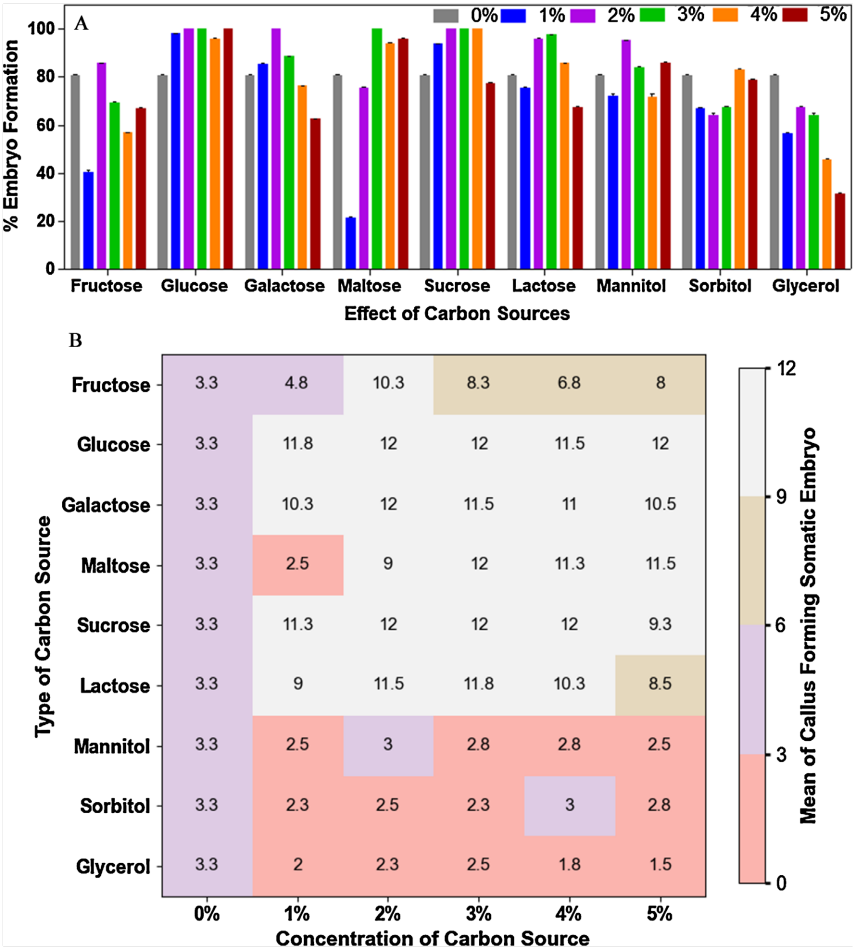

Figure 6. Effect of different carbon sources and their concentration on somatic embryo formation from induced callus in alfalfa cultivar Regen-SY. Vertical lines represent the standard error of the means. **(A)** Percentage of callus-forming somatic embryos; **(B)** Mean number of embryos formed. Alfalfa leaf explants were cultured on Gamborg B5H hormone-free medium supplemented with respective carbon sources, and data were collected three weeks after embryo formation and maturation. Each treatment consisted of 60 explants from four replicate plates, with 12 explants per plate. Vertical lines indicate the standard error of the means. Significant differences between carbon sources ($p < 0.01$) are indicated by different letters above the bars.

Our findings revealed significant differences in embryo formation and maturation across various carbon sources (Figure 5 and Figure 6). The average number of embryos formed per leaf explant varied depending on the type of carbon source: fructose: 4.75 - 10.25 embryos (39.6% - 85.4%), glucose: 11.50 - 12 embryos (80.4% - 100%), galactose: 10.5 - 12 embryos (62.5% - 100%), maltose: 2.50 - 12 embryos (20.8% - 100%), sucrose: 9.25 - 12 embryos (77.1% - 100%), and lactose: 8.5 - 11.75 embryos (67.5% - 97.5%). In contrast, embryo formation and maturation were inhibited at the globular stage in the control medium (no sugar) and media supplemented with sugar alcohols. Embryo formation was minimal in mannitol: 2.5 - 3 embryos (70.8% - 95%), sorbitol: 2.25 - 3 embryos (62.9% - 82.5%), and glycerol: 1.5 - 2.5 embryos (31.5% - 66.7%). Although sugar alcohols did not support embryo

formation, some initial embryo initiation was observed, but further maturation was either limited or absent at all tested concentrations. Similarly, the callus transferred to the control medium exhibited early-stage somatic embryo initiation; however, development was poor, and growth was arrested at an early stage.

3.3. Effect of Carbon Sources on Embryo Conversion

In the embryo conversion study, mature somatic embryos were transferred to a conversion medium supplemented with various carbon sources at different concentrations. This phase aimed to evaluate the influence of different carbon sources on the successful transition of embryos into the development of plantlets in the alfalfa cultivar Regen-SY. Six weeks after transfer under light conditions, the tested carbon sources, such as fructose, glucose, maltose, and sucrose, were anticipated to support somatic embryo conversion and germination. Embryos cultured on these media developed green buds with a higher initial survival rate. However, in media lacking a carbohydrate source (control) or supplemented with mannitol, sorbitol, or glycerol, the green buds gradually withered and failed to regenerate into viable plantlets. Since these treatments did not support plant development and regeneration, they were excluded from the subsequent stages of the experiment.

Mature embryos that successfully converted into plantlets exhibited key developmental traits (Figure 7), including the emergence of visible shoots with true leaves, indicating shoot meristem activation, and the elongation of radicles into functional roots, demonstrating root meristem activation. The formation of a bipolar structure, with both shoot and root systems, serves as an indicator of

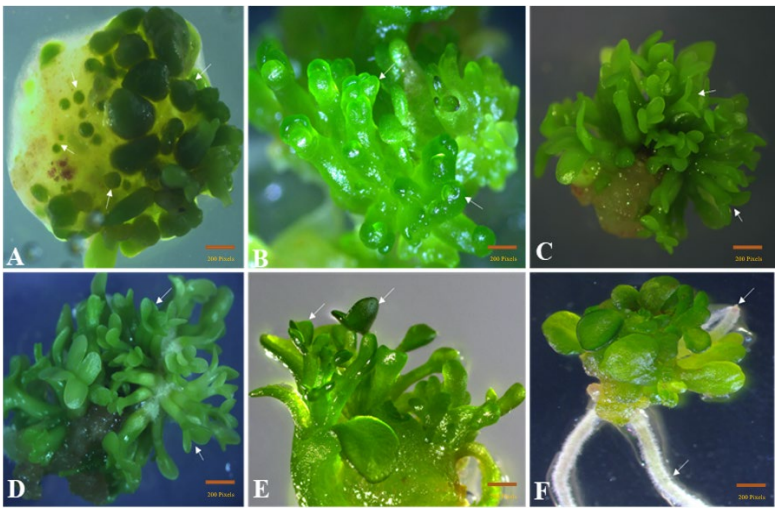

Figure 7. Various stages of somatic embryo development, maturation, and conversion in alfalfa cultivar Regen-SY on maltose-enriched MS medium. **(A)** Conversion of embryogenic cells and pro-embryogenic clumps into the globular-stage somatic embryo; **(B)** Progression of somatic embryos through different developmental stages, from globular to heart-shaped structures; **(C) (D)** Close-up view of embryo development reaching the cotyledonary stage; **(E)** Formation of true leaves following embryo maturation; **(F)** Somatic embryo-derived plantlets exhibiting normal shoots, well-defined tap and lateral roots, confirming the bipolar structure.

successful somatic embryogenesis and subsequent plantlet regeneration.

The results demonstrated that embryos cultured on a maltose-containing medium exhibited high conversion rates, ranging from 234.75 to 767.5 embryos (83.6% - 96.9%), with a remarkable proportion developing into plantlets with enhanced vigor, well-formed shoots, and robust root systems. Medium supplemented with 3% maltose was particularly effective in promoting somatic embryo conversion and plantlet formation (Figure 8), suggesting that it meets the metabolic

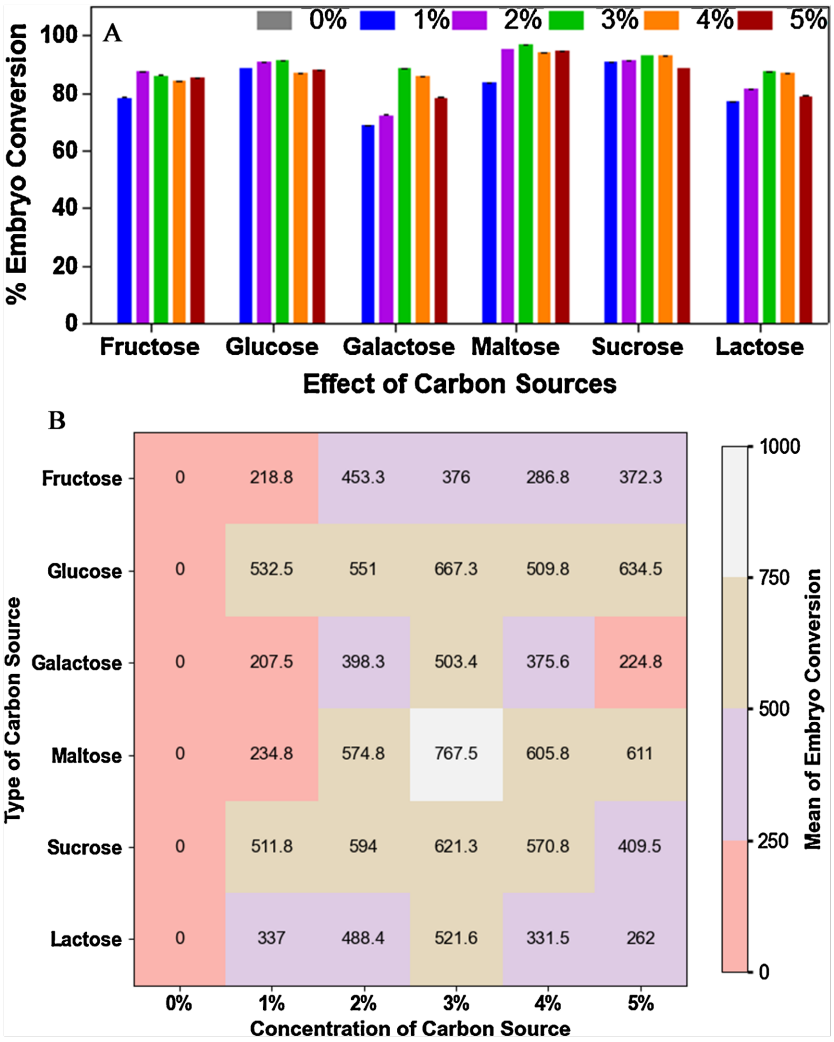

Figure 8. Effect of different carbon sources and their concentrations on somatic embryo conversion from matured embryos in the alfalfa cultivar Regen-SY. **(A)** Percentage of embryo conversion; **(B)** Mean number of converted embryos. Matured embryos derived from alfalfa leaf explants were cultured on MS medium supplemented with various carbon sources, and data were collected three weeks after embryo conversion. Each treatment included 45 converted embryos from four replicate plates, with 9 converted embryos per plate. The medium supplemented with 3% maltose yielded the highest embryo conversion rate. In contrast, media containing mannitol, sorbitol, or control (lacking a carbon source) showed no significant response. Vertical lines represent the standard error of the means, and significant differences between carbon sources ($p < 0.01$) are indicated by different letters above the bars.

demands of embryos during the conversion stage by providing a steady energy supply to support cell differentiation and growth.

In comparison, embryos cultured in the media supplemented with other carbon sources such as fructose: 218.75 - 453.25 embryos (78.4% - 87.3%); glucose: 509.75 - 667.25 embryos (86.9% - 91.2%); galactose: 207.5 - 503.37 embryos (68.3% - 88.8%); sucrose: 409.5 - 621.25 embryos (88.6% - 93.2%); and lactose 262 - 521.63 embryos (76.9% - 87.6%) exhibited the conversion rates. However, media containing mannitol, sorbitol, glycerol, or lacking a carbon source (control) showed no significant response, indicating their ineffectiveness in supporting somatic embryo conversion and plantlet development.

3.4. Effect of Carbon Sources on Alfalfa Regeneration

In a study on whole plant regeneration, germinated somatic embryos exhibiting distinct leaf or shoot primordia were transferred to a shoot elongation medium containing different carbon sources at varying concentrations. The type of carbon source had a significant impact on root and shoot development, as well as overall shoot quality. Nearly all shoots with leafy structures successfully elongated. After nine weeks under light conditions, the tested carbon sources, such as fructose, glucose, galactose, maltose, sucrose, and lactose, effectively supported germination, resulting in normal shoot and root formation.

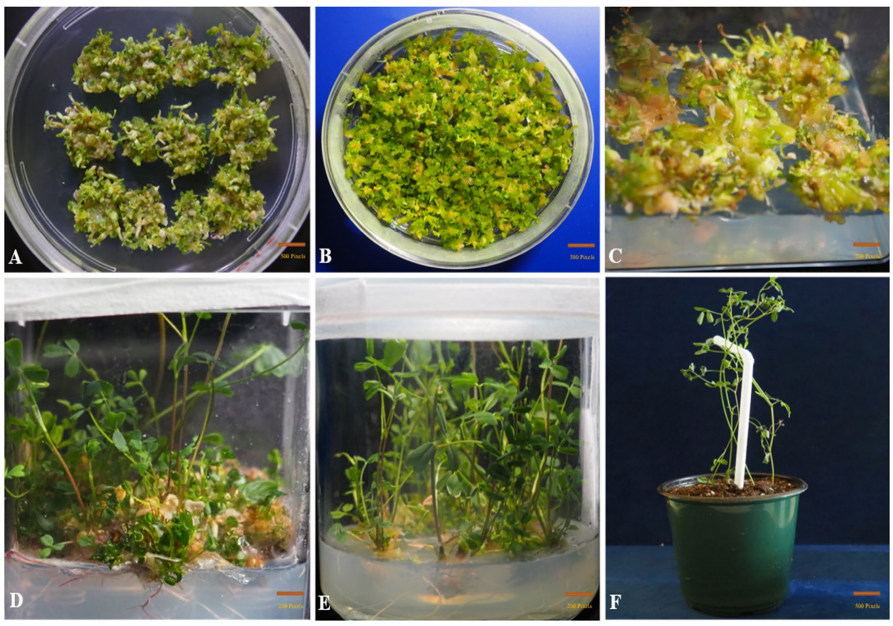

Figure 9. The stages of whole plant development through somatic embryogenesis on a culture MS medium supplemented with 3% maltose. **(A)** Early-stage somatic embryos forming in clusters; **(B)** Actively proliferating somatic embryos densely packed together; **(C)** Somatic embryos transferred to a Magenta box for further development; **(D)** Converted somatic embryos developing into plantlets, exhibiting a bipolar structure with leaf formation and emerging root systems; **(E)** Plantlet growth progressing in a glass jar; **(F)** Mature plant acclimated to *ex vitro* conditions in a pot containing a soil and vermiculite mixture.

Among them, embryos cultured on 3% maltose-containing media produced the highest rate of normal shoot development) (64% - 100%, with 162 - 767.5 plantlets), while other carbon sources produced varying regeneration rates and occasionally led to plantlets with cotyledon-like leaves (Figure 9 and Figure 10). The observed regeneration rates were as follows: fructose (50.2% - 66.6%, 111.5 - 281 plantlets), glucose (51% - 81.9%, 308.75 - 547 plantlets), galactose (50% - 74.5%, 158.6 - 344.5 plantlets), sucrose (60.7% - 85%, 262 - 528 plantlets), and lactose (61.5% - 78.4%, 237 - 507.6 plantlets). Approximately 90% of the elongated shoots efficiently

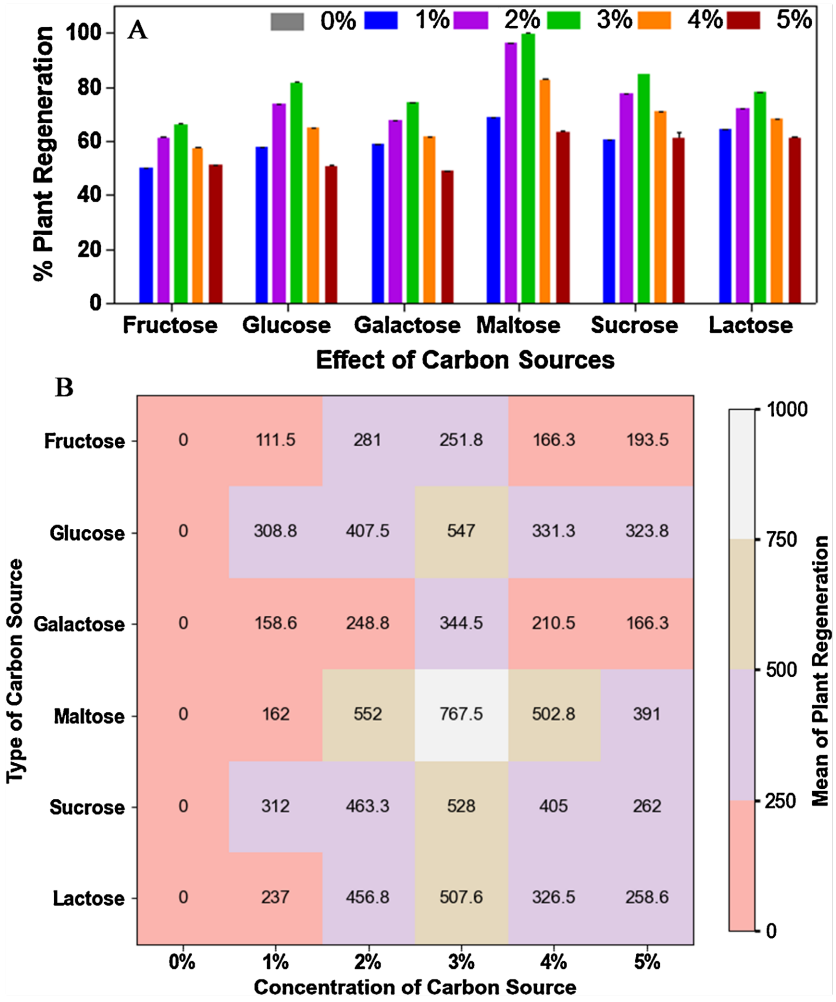

Figure 10. Effect of different carbon sources and their concentrations on plant regeneration through somatic embryos from germinated embryos in the alfalfa cultivar Regen-SY. **(A)** Percentage of Plant regeneration; **(B)** Mean number of regenerated alfalfa plants. Germinated embryos derived from alfalfa leaf explants were cultured on an MS basal medium supplemented with various carbon sources, and data were collected three weeks after embryo conversion. Each treatment included 45 converted embryos from four replicate plates, with 9 converted embryos per plate. The medium supplemented with 3% maltose resulted in the highest rate of regenerated plants. In contrast, media containing mannitol, sorbitol, glycerol, or control (lacking a carbon source) showed no significant response. Vertical lines represent the standard error of the means, and significant differences between carbon sources ($p < 0.01$) are indicated by different letters above the bars.

developed roots within one to two weeks when transferred to the rooting medium. Strong root systems were established by transplanting rooted shoots into half-strength MS salts until new leaves emerged in culture boxes. Depending on the carbon source, 50.2% - 100% (data not shown) of the plants survived in the soil, ultimately developing into fertile plants in the greenhouse (Figure 11).

Carbon Source	Callus Induction	Embryo Formation	Embryo Conversion	Plant Regeneration
Fructose	High	Moderate	Low	Low
Glucose	High	High	High	Moderate
Galactose	High	High	Low	Low
Maltose	High	High	High	High
Sucrose	High	Moderate	High	Moderate
Lactose	High	Moderate	Moderate	Moderate
Mannitol	Low	N/A	N/A	N/A
Sorbitol	Low	N/A	N/A	N/A
Glycerol	Low	N/A	N/A	N/A
Control	Low	N/A	N/A	N/A

Figure 11. Color-coded matrix illustrates the effect of different carbon sources on plant regeneration in alfalfa.

4. Discussion

This study was focused on increasing the efficiency of alfalfa plant regeneration by examining nine different carbon sources across five different concentration levels. Among these, nearly all explants cultured in monosaccharide hexoses (fructose, glucose, galactose) and disaccharides (maltose, sucrose, lactose) developed embryogenic callus. Maltose was found to be the most effective carbon source, yielding the highest rate of embryo formation and maturation. This aligns with the findings of other species, such as apple [22], pea [23], and petunia [24]. Similarly, in alfalfa, Strickland *et al.* (1987) [21] reported optimal embryo yield at higher maltose concentrations (4% - 6%) in clone RA-3, suggesting that maltose's slow hydrolysis supports prolonged nutrient availability. Parrott and Bailey (1993) [33] also confirmed the advantage of maltose in different cultivars of alfalfa, although the response was inconsistent and progression through the developmental stage remained limited. In our study, sugar alcohols or polyols (mannitol, sorbitol, and glycerol) were found ineffective in promoting good quality callus developing into the formation of embryos. It could be due to the osmotic or plasmolytic stress imposed by the compounds. Our observation is consistent with osmotic stress effects reported in other species, such as *Pharbitis nil* [34] and *Oplopanax elatus* [35], where polyols were not efficiently metabolized by the explants leading to carbon starvation in tissues. However, mannitol was effective in *Olea europaea* [7], *Apium graveolens* [25], and *Fraxinus americana* [36], and sorbitol supported somatic embryogenesis in *Zea mays* and *Pyrus pyrifolia*, *Malus domestica* [27], *Elaeis guineensis* [11], and *Prunus persica* [26]. Glycerol also showed promise in different citrus varieties such

as Clementine mandarin and Kutdiken lemon [29].

While monosaccharides and other disaccharides positively influenced embryogenesis in alfalfa, their efficacy was lower than maltose. Despite the lower performance in our study, previous studies have reported their effectiveness in other species. Fructose promoted strong embryogenic responses in *Linum usitatissimum* [37] and *Theobroma cacao* [8] and promoted enhanced shoot formation in *Stevia rebaudiana* [17]. Glucose supported direct shoot regeneration in *Prunus mume* [38], improved shoot production in bitter almond nodal cuttings [18] and *Fagus sylvatica* [39] and rooting in embryogenesis of asparagus [40]. Although galactose—a C-4 epimer of glucose—is reported to be less effective in embryogenesis of species such as *Phaseolus vulgaris* and *Helianthus tuberosus* [13] and *Bauhinia purpurea* L. [41], it interestingly supported citrus species [21]. Similarly, lactose, being uncommon in most of the plant species, supported the production of an acceptable number and quality of somatic embryos in certain conifer species [29], *Bauhinia purpurea* L. [41], and *Hevea brasiliensis* [42]. Sucrose remains a staple in plant tissue culture due to its resemblance to the plant's natural carbohydrate transport system [29]. Although maltose outperformed sucrose in our study, its efficacy has been proven in different varieties of species, such as *Dianthus caryophyllus* [43], *Psidium guajava* [44], and *Picea* spp. [45] [46]. The development of shoots and roots was observed by the ninth week, with the successful regeneration of whole plants by the twelfth week. The consistency was intact as maltose's superiority was observed in the development of plantlets to the whole plants. Similar findings were reported in *Trifolium repens*, *Solanum lycopersicum*, *Theobroma cacao*, and *Pinus* species, where maltose has been identified as an optimal carbon source for supporting plant regeneration [8] [47]. In sugarcane [48] and rice [46], optimal regeneration outcomes were observed using a 3% concentration of maltose, which is similar to our study, where 3% maltose was the most effective, achieving 100% regeneration of the plants from the somatic embryos.

Our findings on advancing the existing somatic embryogenesis protocols not only support mass propagation but also serve as a model system for other leguminous crops to establish the system of tissue culture and genetic engineering. Somatic embryogenesis remains a vital tool, providing solutions where conventional breeding systems are limited due to the complicated genetic makeup of the plants. Future research should focus on elucidating the physiological mechanisms underlying the different responses of various carbon sources, particularly through measurements of osmotic potential. Such insights would clarify if osmotic stress contributed to the ineffectiveness of sugar alcohols. In addition, applying the optimized protocol to different genotypes of alfalfa as well as other legume species would help assess its broader applicability in plant regeneration and genetic engineering systems.

5. Conclusion

Given the critical role of appropriate carbon source selection in optimizing and

facilitating large-scale clonal propagation of alfalfa, we selected different carbon sources to evaluate the optimum type and concentration that enhanced the development of somatic embryos for whole-plant regeneration in alfalfa. This phase of the study was essential for identifying the most effective type and concentration of carbon sources to maximize regeneration yield and contribute valuable insights to the broader research efforts. A strong correlation was observed between somatic embryo formation and its conversion across all tested treatments, indicating the potential involvement of a common intermediate pathway influenced by different carbon sources. Among the evaluated carbon sources, maltose at 3% concentration was the most effective, with 100% plant regeneration. This finding underscores the efficacy of disaccharide-enriched media, particularly those containing maltose, in promoting somatic embryogenesis and successful plant regeneration in alfalfa. The optimized protocol established in this study provides a promising foundation for future genetic transformation efforts, using somatic embryogenesis as a reliable target for both direct and indirect transformation techniques.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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