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EFFECT OF VARYING CONCENTRATIONS OF PHYTAGEL, SORBITOL AND MANNITOL ON HYPERHYDRICITY OF IN VITRO REGENERATED TOBACCO SHOOTS

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ABSTRACT

In vitro regeneration methods that efficiently produce entire plants from single cells are crucial for clonal propagation and genetic engineering. Shoots can be directly induced from mature organs like leaves, bypassing callogenesis in some dicotyledonous plants, like tobacco. Tissue culture exposes plant tissues to specific nutrients, light, and hormones in sterile, *in vitro* conditions to produce genetically identical plants rapidly. Nonetheless, the technique encounters various bottlenecks, notably hyperhydricity or vitrification, a morphological and physiological disorder that involves excessive hydration and abnormal shoot development, causing a glassy, water-soaked appearance due to excessive water uptake. To counter this, we employed RMOP medium with varying phytagel concentrations (1.3g/L, 2.6g/L, 3.9g/L and 5.2g/L) and added different levels of sugar-alcohols e.g., mannitol and sorbitol (250mg/L, 500mg/L, and 750mg/L). Optimal vitrification reduction occurred at 3.9g/L phytagel, though 5.2g/L reduced vitrification but with stunted growth. In alternative media, 2.6g phytagel with 250mg/L mannitol displayed reduced vitrification with robust growth. In conclusion, 3.9g phytagel fosters plant growth, yet its expense raises concern.

***Keywords*:** Tissue culture, Hyperhydricity, Phytagel, Sorbitol, Manitol

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1. INTRODUCTION

Tissue culture is a technique that exposes plant tissues to specific ratios of nutrients, light, and hormones in a sterile, controlled environment, leading to the rapid generation of numerous new plants, each an exact replica of the original mother plant, all achieved in a remarkably short period of time (Rahimi et al., 2023). In essence, in vitro, aseptic culture involves nurturing cells, tissues, organs, or entire plants in a controlled environment with careful attention to nutrition and surroundings. This practice is often employed to generate plant clones (Patil et al., 2021). The specific parameters that provide an optimum nurturing environment ensure that the produced clones precisely replicate their desired genetic traits. The use of plant tissue culture technologies in industrial-scale plant reproduction is widespread as well as for research purposes (Abdalla et al., 2022). Plant tissue culture has gained significant industrial significance in the realms of plant reproduction, disease control, genetic enhancement, and the synthesis of secondary metabolites. Even small pieces of tissue, referred to as explants, have the potential to yield hundreds and even thousands of plants through a continuous and efficient process.

A single explant can quickly produce thousands of plants in a small amount of area and in a controlled environment, independent of the time of year or the weather, this productivity is constant (Khan et al., 2022). The micropropagation technique has proven to be a savior for endangered, threatened, and rare plant species due to its remarkable multiplication rate and minimal requirements in terms of initial plant numbers and space. Furthermore, when it comes to enhancing crops through the creation of somaclonal and gametoclonal variants, plant tissue culture stands out as the most efficient technology in the field (Soumare et al., 2021). Using micropropagation technology, it is possible to isolate important variants from well-suited, high-yielding genetic strains with enhanced disease resistance and increased stress tolerance. This method holds enormous potential for producing plants of excellent quality. Notably, the presence of somaclonal diversity enables certain callus cultures to produce clones with heritable features different from those seen in the parent plants (Rajan and Singh, 2021).

The commercial production of plants using micropropagation techniques offers several distinct advantages when compared to conventional methods such as seed propagation, cuttings, grafting, and air-layering (Meena et al., 2022). Plant tissue culture has a significant impact on both agriculture and industry by providing the necessary plants to

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meet the increasing worldwide demand. It now serves as a crucial tool for modern agriculture as a result of its significant recent contributions to the advancement of agricultural sciences (Coulibaly et al., 2022). Tissue culture enables the creation and dissemination of genetically uniform, pathogen-free plant material (Twaij et al., 2020).

This method of plant regeneration in vitro is widely used as a vital biotechnological tool for reliable clonal reproduction (Henao-Ramírez and Urrea-Trujillo, 2020). This process involves the transformation of somatic cells or tissues into specialized embryos, capable of developing into complete plants without the need for sexual fertilization, as seen in zygotic embryos (Twaij et al., 2020).

It describes the process by which plant organs, such as roots, shoots, and leaves, are produced. These organs may develop directly from the meristem or indirectly from calluses, which are undifferentiated cell masses. Plant regeneration through organogenesis entails the creation of callus and the transformation of adventitious meristems into organs by manipulating the levels of plant growth hormones in the nutrient medium. Skoog and Muller were the trailblazers who showcased that increasing cytokinin-to-auxin ratio encouraged shoot formation in tobacco callus, while an elevated auxin-to-cytokinin ratio triggered root regeneration (Raspor et al., 2021).

A broader range of techniques and technologies, including molecular genetics, recombinant DNA research, genome profiling, gene transfer procedures, and the sterile growing of plant cells, tissues, and organs, include tissue culture techniques as a significant component (Bihari et al., 2022). A noteworthy contribution of this method lies in its revelation of a remarkable property of plant cells known as cellular totipotency, where every living cell within a plant organism possesses the potential to develop into an entire plant (Vir and Kumar, 2021). Micropropagation surpasses traditional crop breeding methods in several aspects. It allows for the swift generation of abundant plant quantities in a short period. The technique offers the advantage of generating numerous plants from a single parent plant's tissue, without harming the parent. Additionally, micropropagation eliminates the risk of interruptions in the growing season, as it can be conducted in a controlled greenhouse environment. Greenhouses allow close monitoring of chemical and physical conditions, preventing production slowdowns due to seasonal variations (Patil et al., 2021).

Micropropagating woody plants presents significant challenges due to the presence of various secondary metabolites. Commonly encountered issues include tissue and media browning, systemic contamination, vitrification, shoot tip necrosis (STN), tissue fasciations, and somaclonal variations. Overcoming these limitations can be achieved through methods such as sterilization facilitated by nanosilver, the development of novel culture media tailored for woody plant propagation, and the identification of numerous new natural plant growth regulators (PGRs) (Sharma et al., 2022).

Phenolic exudates (especially woody perennials) in culture have a problem while establishing explants. These phenolic compounds undergo oxidation, resulting in the formation of a brown substance within the medium, which, in turn, hinders the development of shoots (Permadi et al., 2023). Exudation can be reduced by utilizing antioxidants like ascorbic acid, absorbent materials like activated charcoal, or regular transfers to a new medium (Vala et al., 2020).

Hyperhydricity (HH) is also referred to as vitrification in earlier literature. Tissue becomes waterlogged and translucent due to excess water absorption. This issue is managed by adjusting agar concentration or its source. Hyperhydricity, also known as 'watersoaking' or 'hyperhydration,' represents a prevalent issue in plant tissue culture, especially in micropropagation. It constitutes a spontaneous and intricate disorder that impacts plants cultivated *in vitro*, arising from a multitude of factors during the culture process. This leads to irregular shoot growth along with anatomical and physiological changes. Hyperhydric shoots exhibit visible deformities like thick, brittle, curled, and translucent leaves (Bethge et al., 2023). As suggested by the name, hyperhydric plants have excessive water content. They are incapable to manage water balance and accumulate it (Hernandez-Apaolaza, 2022). High humidity and the type of gelling agent significantly contribute to HH (Al-Mayahi and Ali, 2021). When *in vitro* cultured plantlets are moved to soil, they fail to survive due to leaf changes like yellowing, swelling, glassiness, and curling often tied to reduced photosynthesis (Maurya et al., 2023). Accumulated gases like ethylene and CO2 also play a role in causing HH (García-Ramírez, 2023). Microscopic analyses have revealed that the mesophyll of hyperhydric plant leaves contains significant intercellular gaps, a reduced number of palisade cells, and underdeveloped epicuticular wax layers (Chen et al., 2020). Additional abnormalities, such as a deficiency in chlorophyll, may also be observed, low lignification and malformed stomata have been reported (Din et al., 2020). Hyperhydricity occurs across a diverse array of plant species, spanning from garlic to apple. Several strategies to mitigate hyperhydricity include employing containers that facilitate proper gaseous exchange, adjusting agar concentrations, and modifying the nitrate-to-ammonium ion ratio. It's important to note that hyperhydricity is not confined solely to shoots; certain callus types, appearing yellowish and hyperhydric, may also be deemed vitreous (Mohamed et al., 2023). Other factors contributing to HH include ethylene and cytokinins (Jan et al., 2021). Two water accumulation sites are proposed: one in the symplast due to deficient cellulose and lignin and another in the apoplast, the cell wall continuum and intercellular spaces (Kassemeyer et al., 2022). Apoplastic water accumulation disrupts gas exchange, slowing gas diffusion compared to air (Stegner et al., 2023), potentially causing physiological disorders. Despite complexities, a clear link between symplastic water excess and HH symptoms remains uncertain (Kassemeyer et al., 2022).

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2. MATERIALS AND METHODS

2.1. Media Composition and Preparation

For the preparation of RMOP Sucrose (30g/L), MS salts (4.33g/L) and Thiamine HCl (1mg/L) were weighed in required quantity and dissolved in 500-600mL of double distilled deionized water. Moreover, NAA (1mg/L) and BAP (0.1mg/L) solutions were prepared for use by stock solutions. Volume was made up to 1 liter by the addition of double distilled deionized water. Different concentrations of phytagel (1.3g/L, 2.6g/L,3.9g/L, and 5.2g/L), sorbitol (250mg/L ,500mg/L & 750mg/L), and mannitol (250mg/L, 500mg/L &750mg/L) added in above mentioned RMOP medium. Finally, pH was adjusted to 5.8 and the required amount of phytagel was added before autoclaving at 121ºC for 15 minutes. For culturing, healthy lush green leaves from *in vitro*-grown tobacco plants were used for these experiments. These leaves were carefully dissected into 5-6mm^2 using a sterile scalpel blade and cultured onto modified RMOP media. Tissues from regenerated shoots were excised from all media and preserved (in 70% ethanol) for anatomical studies.

2.2. Sectioning, Staining and Microscopy

A potato slice (used as a base) has been taken, and a hole was made in it with a needle. A shoot was inserted into the hole and leveled with a sharp blade. For the midrib of the leaf, a potato slice was cut, and a leaf was placed in it; the section was then cut with a sharp blade. The section was cut with a sharp blade in water. Subsequently, a good section was selected, and the staining process was initiated. Thin sections were chosen and immersed in 30%, 50%, and 70% ethanol for 15 minutes respectively. The sections were placed in Safranin (1g safranin dissolved in 70% ethanol) for 15 minutes. Next, they were transferred to 90% ethanol for 5 minutes, followed by a 1-minute immersion in fast green (1g dissolved in 90% ethanol). After washing with 100% ethanol to stop staining, the sections were dehydrated and treated with xylene. A slide was prepared with one drop of Canada balsam, the section was mounted using a needle, and the cover slip was carefully placed on top. A compound microscope with 100 X magnifying power has been used for the anatomy of tissues.

3. RESULTS

In vitro regeneration and micropropagation techniques have found extensive application in facilitating the rapid clonal propagation of numerous economically significant plant species. But, the success of these methods depends on making many healthy plants at a low cost and with high survival rates (Arora et al., 2022). Understanding how these plants change during growth can improve these techniques. There are problems, however, the tissue culture conditions conducive to accelerated shoot growth and multiplication frequently lead to the development of structurally and physiologically aberrant plants (Bidabadi and Jain, 2020). A significant concern in this regard is hyperhydricity, a condition where plants, often referred to as vitrified due to their glassy appearance, exhibit thick, rigid stem leaves that are prone to breakage. These plants are marked by reduced protein and chlorophyll levels, elevated water content, an altered ion composition, high fresh weight and low dry weight, as well as enlarged hypertrophic cells with expanded vacuoles and intercellular spaces (Kemat et al., 2021).

The present work was conducted in Transgenic Lab at Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture Faisalabad. We have observed the problem of hyperhydricity in the *in vitro* cultures of tobacco available. The *in vitro* grown tobacco plants showed stunted growth, glassy appearance, and light green foliage due to lower chlorophyll contents as depicted in Fig. 1. Hence, we will investigate the causes and cure of hyperhydricity in this article. We conducted an experiment using various regeneration media containing different levels of the thickening agent phytagel, and two osmolytes—mannitol and sorbitol—to assess their effects.

3.1. Using Different Concentrations of Phytagel

In plant tissue cultivation, a solid or semi-solid nutrient medium is commonly used. Different thickening agents, like Gel-Gro®, Gerlite®, and Phytagel®, are used to solidify these media. These agents are polysaccharides produced by bacteria and are clean from contaminants. We placed healthy tobacco leaves grown *in vitro* on various levels of phytagel to study how different concentrations affect the reduction of hyperhydricity. The lowest phytagel concentration (1.3 g/L) had the highest vitrification percentage (90%), as seen in Grpagh 1. Regenerated shoot leaves were glossy and crisp. There was large leaf growth due to too much water. Microscope views showed abnormal cell growth with big spaces (Fig. 2.1-A). Chlorophyll was low, making leaves light green (Fig. 2.2-A). Using 2.6g/L phytagel resulted in lower vitrification (70%). Leaves on regrown shoots were less shiny and more vitrified. Leaves were small, but green with better chlorophyll (Fig. 2.2-B). Experiments with 3.9g/L and 5.2g/L phytagel showed the lowest vitrification percentages (25% and 8% respectively) as shown in Fig. 2.2-C & D. 3.9g/L phytagel worked best in controlling vitrification, reducing it by 65% and promoting good plant growth. On the other hand, 5.2g/L phytagel lowered vitrification to 88%, but greatly reduced plant growth by lowering leaf count and size.

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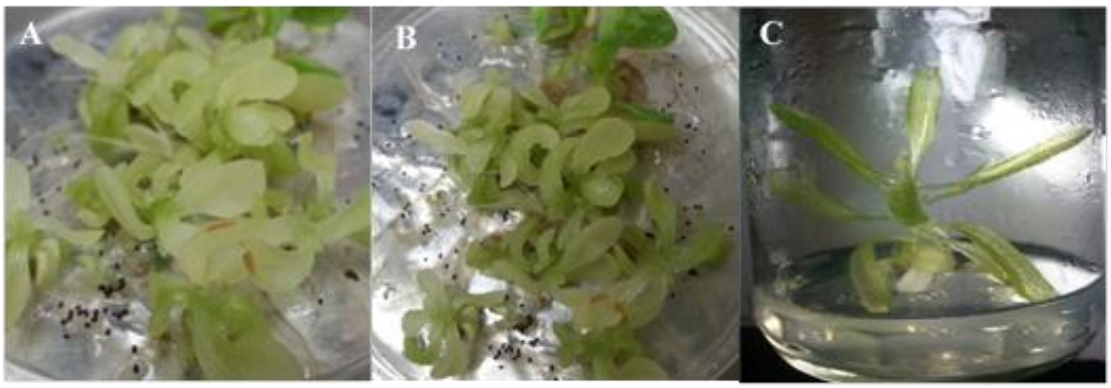

Fig. 1: Typical morphology of hyperhydric tobacco plants: A, B & C shows Glassy appearance, light green colour and stunted growth can be easily observed.

Fig. 2: Effects of different concentrations of phytagel on tobacco plants. A: Phytagel concentration is 1.3 g/L. The figure shows that plants are hyperhydric, having a high amount of water and glassiness on their leaves and shoots. B: Phytagel concentration is 2.6g/L which indicates that the extent of vitrification is less in comparison with A. C: Phytagel concentration is 3.9g/L, plants are less vitrified and growth is significantly high. D: Phytagel concentration is 5.2g/L. This concentration is more than optimum so it results in stunted growth of plants but on the other hand, vitrification is also reduced greatly.

Fig. 3: Effects of different concentrations of Mannitol on tobacco plants. A: Mannitol concentration is 250mg/L, plants are less hyperhydric and representing excellent growth along with visible green leaves. B: Mannitol concentration is 500mg/L that indicates higher vitrification with lower plant tissue proliferation. C: Mannitol concentration is 750mg/L that stipulate the relatively low vitrification. However, plant growth is stunted

3.2. Different Concentrations of Mannitol used

In the second experiment, RMOP medium was supplemented with 250mg/L, 500mg/L and 750mg/L of mannitol to investigate its effect on minimizing the extent of vitrification. In the second experiment, we added different amounts of mannitol to the RMOP medium to see its effect on reducing vitrification. We used 250mg/L, 500mg/L, and 750mg/L of mannitol. The first medium with 250mg/L mannitol showed the best growth, with only 14% of shoots and leaves vitrified. The leaves had good chlorophyll development and more biomass. The second medium with 500mg/L mannitol had more vitrified shoots (62%) and fewer leaves, indicating it wasn't good for plant regeneration due to hindered growth. The third medium with 750mg/L mannitol had higher vitrification (43%). So, the best combination was 250mg/L mannitol with 2.6g/L phytagel for high regeneration and low vitrification (Graph 2).

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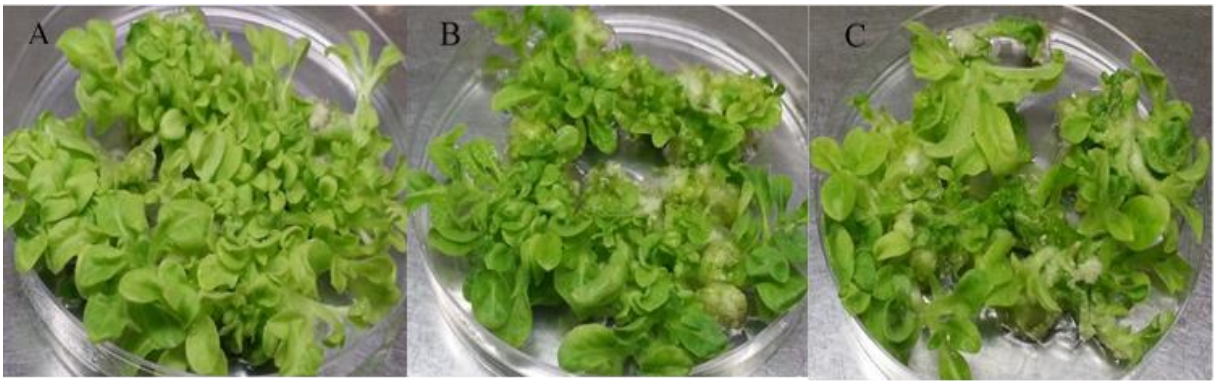

Fig. 4: Effects of different concentrations of sorbitol on tobacco plants. A: Sorbitol concentration is 250mg/L and plants are less hyperhydric with substantial plant growth and fully grown green leaves. B: Sorbitol concentration is 500mg/L, here higher vitrification with lower plant tissue proliferation can be observed. C: Sorbitol concentration is 750mg/L, and the vitrification is relatively low however plant growth has also reduced significantly.

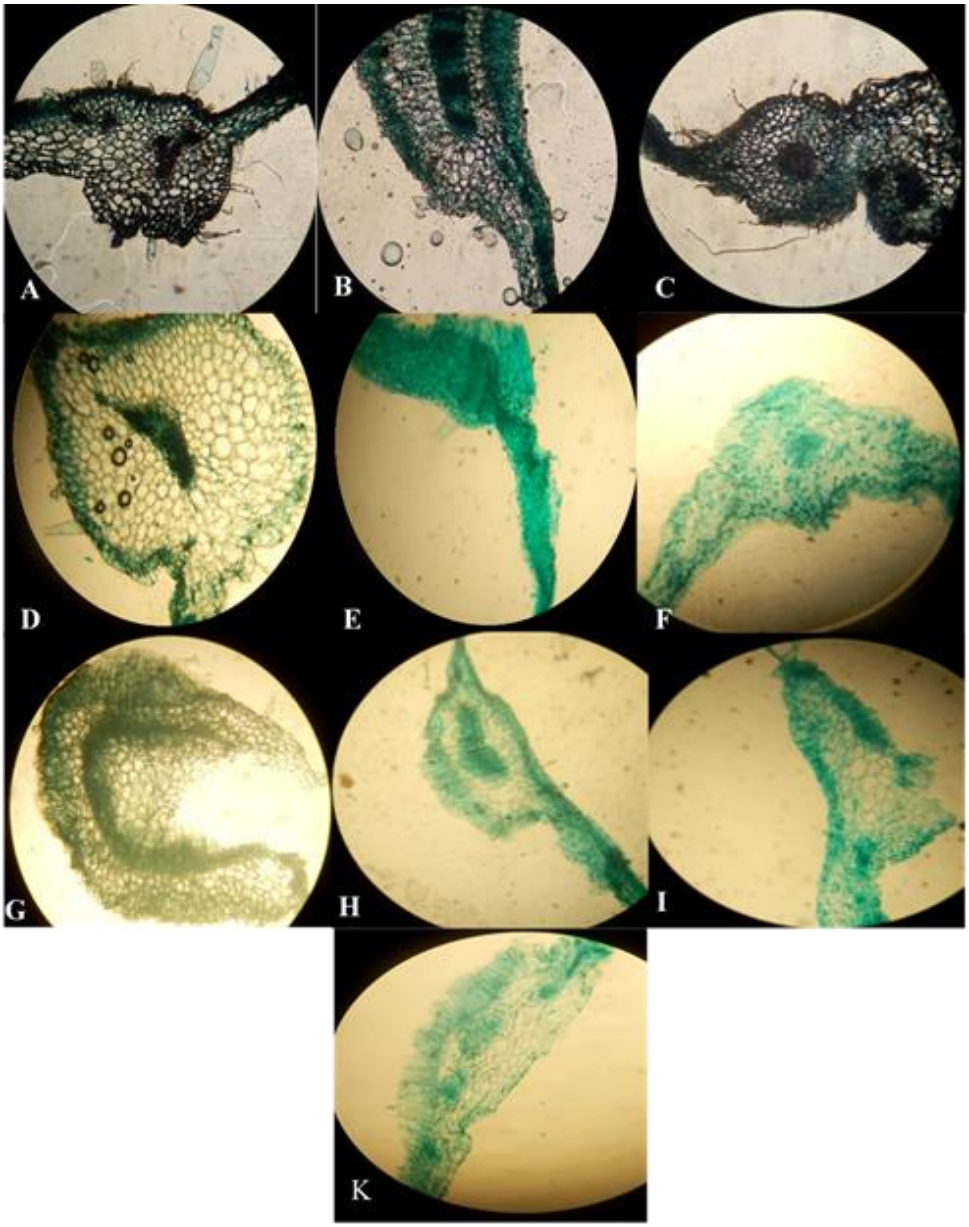

Fig. 5: Micrographs of transverse sections of hyperhydric Tobacco leaves stained with basic safranin-fast green combination. Variation in leaf anatomy is the function of different concentration of Phytagel, Sorbitol and Mannitol. A: Phytagel 5.2g/L, B: phytagel 3.9g/L, C: Phytagel 1.3g/L. D: Sorbitol 750mg/L, E: Sorbitol 500mg/L, F: Sorbitol 250 mg/L, G: Mannitol 750mg/L, H: Mannitol 5000mg/L, I: Mannitol 250mg/L, J: Phytagel 2.6g/L. Magnification: 100X

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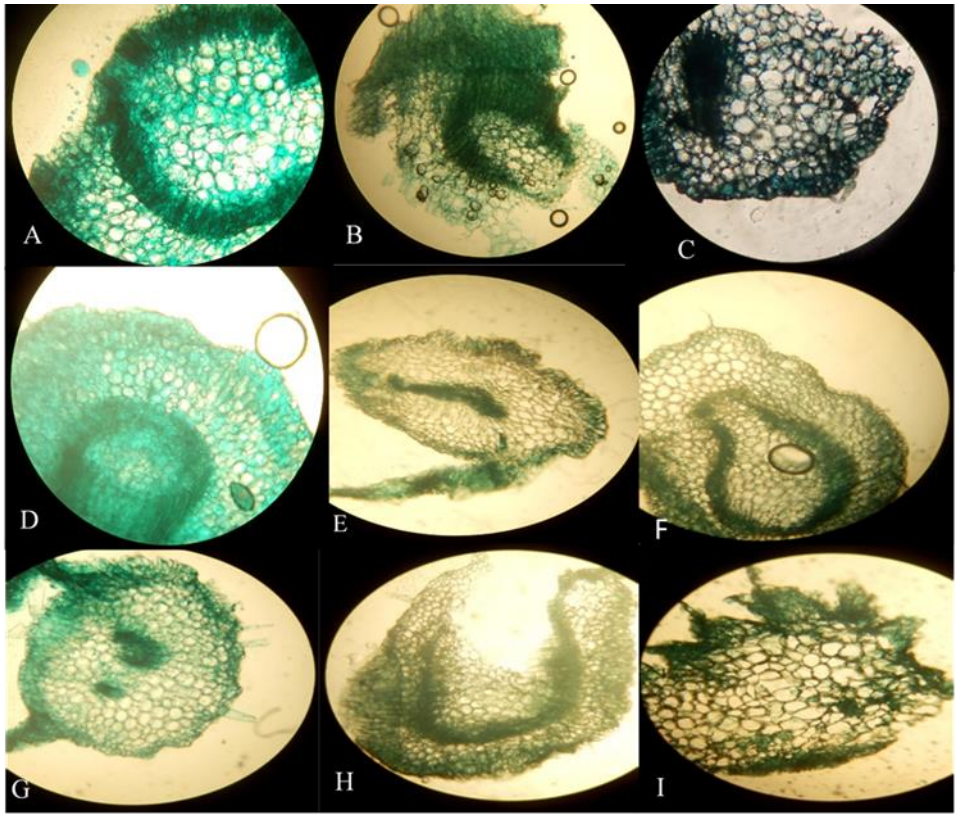
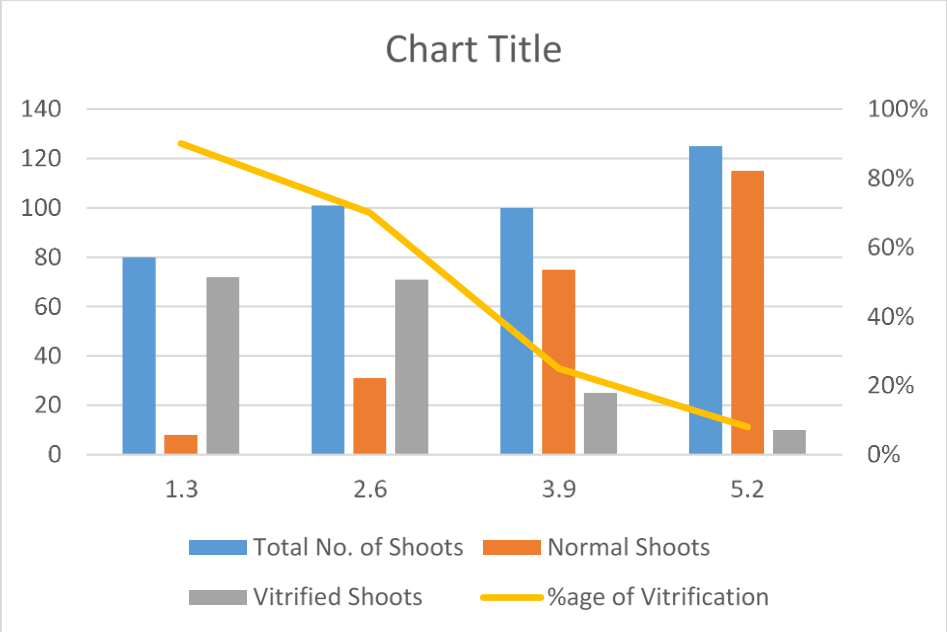


Fig. 6: Micrographs of transverse sections of hyperhydric Tobacco stems stained with basic safranin-fast green combination. Variation in stem anatomy is the function of different concentration of Phytagel, Sorbitol and Mannitol. **A:** Phytagel 5.2g/L, **B:** phytagel 3.9g/L, **C:** Phytagel 1.3g/L, **D:** Sorbitol 750mg/L, **E:** Sorbitol 500mg/L, **F:** Sorbitol 250 mg/L, **G:** Mannitol 500mg/L, **H:** Mannitol 250mg/L, **I:** Phytagel 2.6g/L. Magnification: 100X



Graph 1: Effect of various phytagel concentrations on hyperhydric tobacco plants.

3.3. Different Concentrations of Sorbitol used

In the third experiment, we added varying amounts of sorbitol to the RMOP medium to assess its impact on reducing vitrification. Concentrations of 250mg/L, 500mg/L, and 750mg/L were used. The first medium with 250mg/L sorbitol exhibited robust growth, yielding healthy green leaves and the least vitrification (14%) in shoots and leaves. Chlorophyll development was sufficient, enhancing biomass production. Conversely, the second medium with 500mg/L sorbitol showed higher vitrification (62%) in shoots, indicating hindered growth and regeneration. The third medium with 750mg/L sorbitol also displayed increased vitrification (43%) as shown in Grapgh 3. Thus, the optimal combination was 250mg/L sorbitol with 2.6g/L phytagel for producing more regenerants with minimal vitrification.

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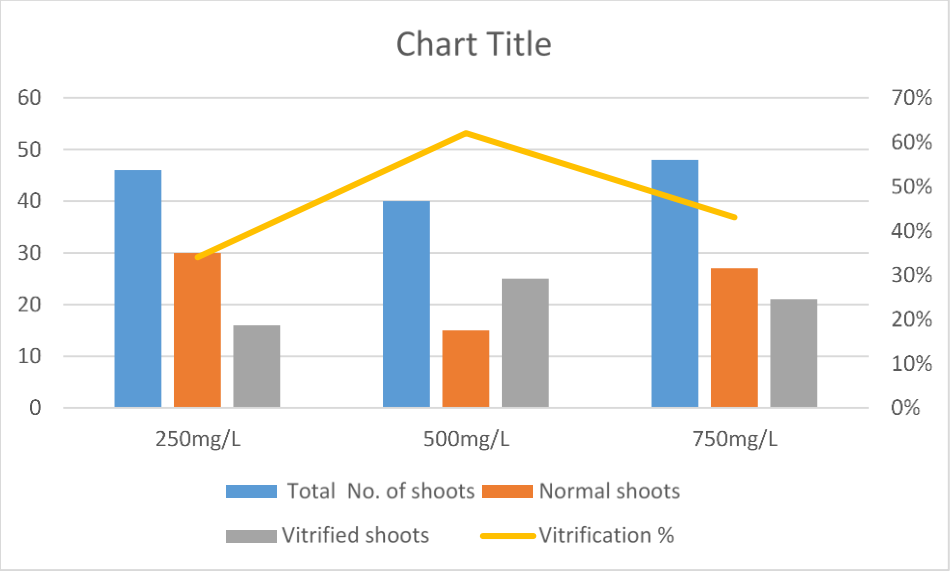
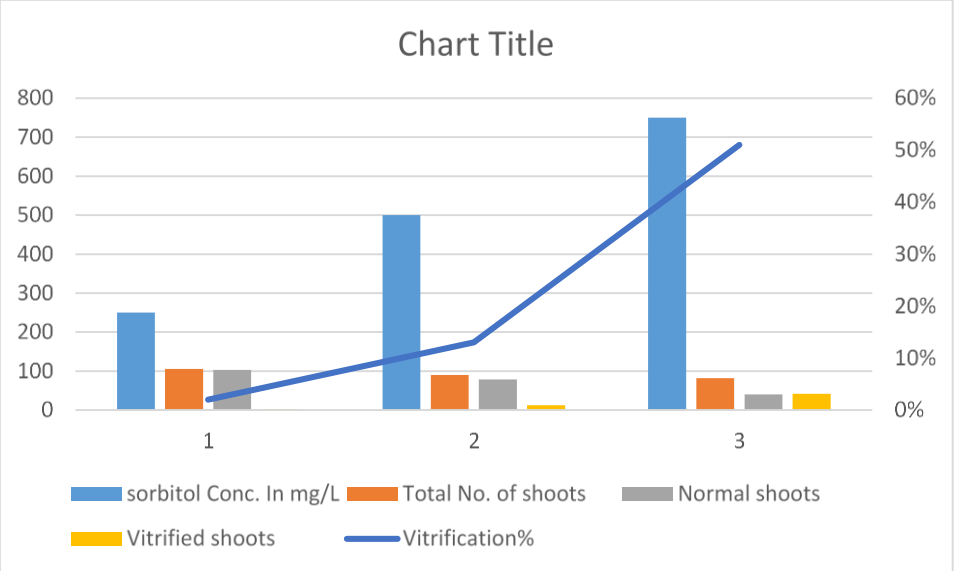


Graph 2: Effect of various Mannitol concentrations on hyperhydric tobacco plants.



Graph 3: Effect of various phytagel concentrations on hyperhydric tobacco plants.

3.4. Anatomical Observation

An unorganized spongy mesophyll with large intercellular spaces has been observed in hyperhydric leaves. Microscopic examination revealed an asymmetrical leave shape with reduced leaf diameter and a progressive reduction in the size of palisade cells, resulting in a nearly imperceptible palisade layer (Fig. 5). The diminishing palisade cells are indirectly indicating that this leave section is abundantly occupied with spongy mesophyll cells (Fig A). Similar observations were obtained for (Fig B) except palisade layer is less reduced. By using 1.3g/L phytagel, an irregular shape with enlarged cell size and diluted cytoplasm (Fig C). These were obtained in comparison with (Fig K) which represents intact upper and lower epidermis, a defined palisade layer and spongy mesophyll cells. Sorbitol and mannitol (750mg/L) have shown similar effects as there is increased cell volume, more intracellular space, and decreased dry weight (Fig D and G). by using sorbitol and mannitol 500mg/L, curly leaves with unclear differentiation of spongy and palisade cells (Fig E) and compact palisade and spongy cells can be visualized (Fig F). Differentiated palisade layer and spongy mesophyll layer can be visualized which is much the same as using phytagel at 2.6mg/L concentration. (Fig F and I).

Microscopy of tobacco stems provides more accurate details about hyperhydricity with varying concentrations of phytagel, sorbitol, and mannitol (Fig 6). In (Fig A), a clear ring of xylem and phloem is found along with more intracellular spaces in the cortex. Moreover, the hypotrophy of the pith can be observed. In (Fig B, C, D, E, G, and I), hypertrophy of cortical region as well as undifferentiated xylem and phloem can be observed. A clear epidermis with an unremarkable cortex and vascular bundles is an indication that using 250mg/L sorbitol has an advantageous role in reducing hyperhydricity (Fig F). Almost identical results were obtained using mannitol 250mg/L (Fig H).

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4. DISCUSSION

The current study was performed to investigate the effects of different concentrations of phytagel, mannitol, and sorbitol on *in vitro-grown* tobacco plants. At 1.3g concentration of phytagel results showed that the plants were more vitrified and due to less quantity of phytagel there was more water availability for plants thus more nutrient availability of plants present that is why shoots became highly vitrified. While at 2.6g which is used as a standard concentration of phytagel in one lite, results also showed vitrification while at 3.9g the vitrification was less and the growth of leaves was better having good results in this medium than in the other two above-mentioned. Thus, this experiment obtained good results at 3.9g and 5.2g, but by using this concentration of phytagel the tissue culture technique will become a more costly technique. Sorbitol and mannitol are inexpensive alternatives among studied gelling agents, and their use may reduce the costs of tissue culture. Sorbitol and mannitol are alcoholic sugars. Our results coincide with (Polivanova and Bedarev, 2022) explain the effect of different gelling agents on hyperhydricity. Lower concentration of sorbitol and mannitol have a beneficial effect on reducing hyperhydricity as it enhances callus growth. While, with elevating concentrations of Sorbitol and Manitol, hyperhydricty increased so it is clear that increasing the sorbitol and mannitol concentrations suppresses the growth on fresh and dry weight as reported by (Kishor, 1987).

To conclude, hyperhydric leaves seem to possess a spongy mesophyll characterized by irregular intracellular spaces (Picoli et al., 2001). The hypertrophy of cortical and pith parenchyma and the hypolignification of the vascular system were exhibited by the hyperhydrated tobacco stems (Marques et al., 2021).

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