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Encapsulation Technique and Chemical Analysis to Improve the Production of Mulberry (*Morus alba*) var. kanva2

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ABSTRACT

Encapsulation technology is a versatile technique widely applied in plant biotechnology for the conservation and propagation of plant material. *In vitro* preservation of the Mulberry (*Morus alba*) variety kanava 2 was evaluated using encapsulation techniques combined with slow-growth storage. Shoot tips and nodal segments derived from *in vitro* cultures were encapsulated in calcium-alginate beads. An ion exchange process lasting 20 minutes was determined to be the most effective for forming well-structured beads. The encapsulated and non-encapsulated samples were stored at 4°C, either in darkness or light. After nine months of storage in cold, dark conditions, encapsulated shoot tips exhibited the highest regrowth rate at 83.3%. Similarly, encapsulated nodal segments achieved a regrowth rate of 55.6% under identical conditions. Overall, the encapsulated samples demonstrated superior regrowth potential compared to non-encapsulated samples following storage. Chlorophyll a and chlorophyll b content was determined. This study aimed to optimize the parameters for encapsulating shoot tips and nodal segments from in vitro cultures of selected *Morus species* using sodium alginate gel.

Keywords: Mulberry, alginate bead, in vitro, chlorophyll, Encapsulation.

Introduction

Mulberry (*Morus spp*) is a versatile tree primarily grown for its leaves, which are used to feed silkworms. Certain varieties are also prized for their sweet, edible fruits or valuable timber (Vijayan *et al.,* 2011a, 2012). Mulberry is a crucial crop for sericulture, traditionally propagated through grafting and stem cutting methods. However, these conventional propagation techniques face several challenges, including dependence on environmental conditions and the physiological state of the cuttings. Synthetic seed technology offers a promising alternative for large-scale, cost-effective clonal propagation and the conservation of mulberry germplasm. The concept of synthetic seeds was first introduced as a potential solution to these issues and submitted by Murashige (1978), Synthetic seeds, also referred to as "synseeds," are defined as "encapsulated single somatic embryos." This process involves encapsulating adventitious buds or somatic embryos, which are induced through tissue culture, enabling them to function like seeds. These artificial seeds can be sown directly in the field, where they will grow, thus offering a convenient and efficient method for clonal propagation and germplasm conservation. (Desai *et al.,* 2022) Although the development of synthetic seeds is anticipated for a variety of crops, it has only been successfully achieved so far with alfalfa **(**Redenbaugh *et al.,* 1986, 1987), carrot **(**Kitto and Janick, 1985a, 1985b**)**, celery **(**Redenbaugh *et al.,* 1986 and Sakamoto, 1990**)** and lettuce **(**Sakamoto, 1990**)**. Encapsulation technology is a cutting-edge approach that enhances the management of plant material derived from in vitro cultures. It is particularly beneficial for the mass production of high-quality plants and for the preservation of germplasm. By encapsulating plant material, this technology ensures that the material remains viable for longer periods, facilitating large-scale propagation and

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aiding in conservation efforts. **(**Standardi and Micheli, 2013**)**. Encapsulation is a promising biotechnological method used for the conservation, propagation, and transport of valuable plant species such as mulberry (*Morus spp*.). The process involves creating synthetic seeds by encasing somatic embryos, shoot tips, or nodal segments within a gel matrix made of sodium alginate and calcium chloride. This technique offers several benefits: it protects the plant material physically, helps maintain its viability, and simplifies its handling and storage, making it an ideal solution for large-scale propagation and long-term conservation (Bhojwani and Razdan, 1996 and Micheli *et al.,* 2017). In mulberry cultivation, encapsulation has been investigated as a promising technique for both germplasm conservation and large-scale propagation. Research indicates that encapsulated nodal segments of mulberry maintain high levels of viability and regeneration capacity. This method proves to be highly effective for the long-term storage and transport of mulberry under non-sterile conditions, offering significant advantages for preserving genetic material and facilitating its propagation in various environments (Ghosh *et al.,* 2020). Encapsulation also plays a key role in safeguarding plant material against mechanical damage and microbial contamination during transport. This method allows for the safe exchange of genetic resources between research institutions while preserving genetic integrity. By ensuring the stability of valuable mulberry genotypes, encapsulation supports their continued use in breeding and cultivation initiatives, further advancing mulberry propagation and conservation efforts. (Singh *et al.,* 2012). With continued refinement, encapsulation could become a pivotal technique in promoting sustainable mulberry cultivation and supporting the silk industry. This method is particularly beneficial for plants like mulberry, where encapsulated nodal segments or embryos maintain strong regenerative capabilities. Such a strategy not only enhances research efforts but also offers practical applications in plant biotechnology, making it an important tool for advancing mulberry propagation and conservation. (Singh *et al.,* 2012; Ghosh *et al.,* 2020). The report also examined the morphogenic response of the encapsulated explants under various conditions. This encapsulation technique holds great potential for enhancing mulberry's regenerative capacity, offering promising opportunities for genetic conservation and applications in biotechnological advancements related to crop production.

2. Materials and Methods

2.1. Plant Material

Nodal segments and shoot tips of mulberry (*Morus spp*.) variety kanava 2 growing in the ex perimental farm of the Sericulture Research Department (SRD), Plant Protection Research, Institute Agriculture Research Center, Giza, Egypt, were used as source materials. were obtained from healthy, disease-free plants grown under controlled conditions. The explants were surface sterilized by immersing them in a 70% ethanol solution for 2 minutes, followed by treatment with a 0.1% mercuric chloride ($HgCl_2$) solution for 5 minutes, and then rinsed thoroughly with sterile distilled water.

2.2. Culture Media

Murashige and Skoog (MS) medium was used for in vitro propagation (Murashige & Skoog, 1962), supplemented with 3% sucrose and 0.7% agar. The medium was autoclaved for 20 min at 121 °C. The cultures were maintained at 23 ± 1 °C in a growth chamber under a 16 h photoperiod with light intensity 60 µmol·m−2·s−1 (standard culture conditions)

For shoot induction, the medium was supplemented with 1.5 mg/L BAP (Benzyl Amino Purine), For rooting, 1 mg/L NAA (Naphthalene Acetic Acid) was added to the medium.

2.3. Sodium Alginate Solution Preparation

A 3% (w/v) sodium alginate (Sigma-Aldrich, USA) solution was prepared by dissolving sodium alginate in sterile distilled water under constant stirring at room temperature. The solution was filtered to remove any impurities and used immediately for encapsulation. All operations were performed under sterile conditions to achieve polymerization and prepare beads of an ideal shape and size with a uniform texture.

2.4. Encapsulation Procedure

In the first experiment, different periods (10, 20 or 30 min) for Na^+/Ca^{2+} ion exchange in the calcium chloride solution were tested. After each incubation period in the complexing agent, the encapsulated explants were retrieved and rinsed three times in sterile distilled water to remove traces of calcium

chloride. The regrowth of the explants was then assessed as described below. with nodal segments sucked into a micropipette with sterile plastic tips and dropped into MS liquid medium supplemented with 100 mM calcium chloride ($CaCl_2.2H_2O$) for complexation. All operations were performed under sterile conditions. To achieve polymerization and prepare beads of an ideal shape and size with a uniform texture, different periods (10, 20 or 30 min) for Na^+/Ca^{2+} ion exchange in the calcium chloride solution were tested in the first experiment. After each incubation period in the complexing agent, the encapsulated explants were retrieved and rinsed three times in sterile distilled water to remove traces of calcium chloride.

2.5. Storage and Maintenance

The regrowth of the explants was then assessed as described below. The following experiment was carried out on both encapsulated, with 20 min of ion exchange time, and unencapsulated explants, which were placed in 90 cm diameter Petri dishes containing semisolid, hormone-free MS medium and held at 4 °C in a growth chamber in darkness or in light (30 molm 2s 1,8h photoperiod) for 3, 6 or 9 months of storage. Regrowth of the explants (encapsulated and unencapsulated) was evaluated for bead incubation time and each cold storage period after transferring them onto proliferation medium for 17 days, under standard conditions. The regrowth ability of encapsulated explants was determined as the time required for the shoot to appear and break through the gel (Standardi *et al.* 2013). Ten explants of each type were placed in a Petri dish, and dishes were replicated three times for each incubation time or treatment combination by storage period. These stored beads were later used for regeneration studies or further analytical processes, making this method invaluable for the conservation and propagation of plant germplasm.

2.6. Regeneration and Viability Assessment

Following encapsulation, the nodal segments and shoot tips were cultured in Murashige and Skoog (MS) medium supplemented with appropriate growth regulators (1.5 mg/L BAP and 1 mg/L NAA) for shoot induction and rooting. Regeneration capacity and viability were assessed, based on the number of regenerated shoots and root formation.

2.7. Chemical analysis

2.7.1. Determination of Chlorophyll

The chlorophyll content in leaves was estimated using Arnon's method (1949). Using the absorption coefficients, the amount of chlorophyll was calculated. 1 g of the leaf sample was weighed and mixed thoroughly with 20 ml of 80% acetone. The solution was centrifuged at 5000 rpm for 5 minutes and the supernatant was transferred to 100ml volumetric flask. The procedure was repeated until the residue was colorless. The mortar and pestle were washed thoroughly with 80% acetone and the washings were collected in the volumetric flask. The volume was made up to 100 ml with 80% acetone. The absorbance of the solution was recorded at 645, and 663 nm against the solvent (80% acetone) blank. The amount of chlorophyll present in the extract mg chlorophyll per g tissue was calculated using the following equations.

Mg chlorophyll a/g = 12.7 (A663) – 2.69 (A645) x V/1000xW
Mg chlorophyll b/g = 22.9 (A645) – 4.68 (A663) x V/1000xW
Mg total chlorophyll = 20.2 (A645) + 8.02 (A663) x V/1000xW
Where A= Absorbance at specific wavelength
V= Final volume of chlorophyll extract 80% acetone
W= Fresh weight of tissue extracted

2.8. Statistical Analysis

The average regrowth time was calculated as follows: (NxTx)/number of developed shoots; where Nx is the number of developed shoots within consecutive intervals of time, Tx is the number of days between the beginning of the test and the end of the specific time interval. Data on regrowth were recorded and presented as means with standard error of the mean (SEM). Significant differences among means were analyzed following analysis of variance using Duncan's Multiple Range Test at $p < 0.05$. Statistical analysis of percentages was carried out by a non-parametric Chi square test (p 0.05) for

pairwise comparisons. All statistical tests were performed with Systat 13 (Systat Software, Inc., San Jose, CA, USA).

3. Results

The findings confirm that mulberry plants derived from tissue culture exhibit superior health and are free from diseases, having been germinated in strictly sterile environments comparing field type. This process ensures the production of uniform and high-quality plants suitable for large-scale propagation, as depicted in Figure 1.

Fig. 1: Mulberry (*Morus spp.)* variety kanava 2 as a source of explants was obtained and grown under controlled conditions. For shoot induction, the medium MS was supplemented with 1.5 mg/L BAP (Benzyl Amino Purine), and for rooting, the medium MS was supplemented with 1 mg/L NAA (Naphthalene Acetic Acid). Culture media were gelled by 0.7% agar.

The optimal ion exchange time for encapsulating mulberry shoot tips and nodal segments using 3% sodium alginate and 100 mM calcium chloride was identified as 20 minutes (Table 1). This duration was most effective for creating smooth, manageable beads around the explants. More than 92% of beads containing shoot tips and 83% of those with nodal segments regrew successfully within the shortest average time-10 days for shoot tips and 20 days for nodal segments. Shorter or longer complexation times resulted in lower regrowth percentages; for example, beads formed after 10 minutes were too soft to handle, while those formed after 30 minutes were more stable but showed slower regrowth. During encapsulation, shoot tips and nodal segments were immersed in sodium alginate solution to ensure full coverage of the tissues (Figure 2) These explants were then transferred to a sterile 0.1 M calcium chloride solution for 20 minutes to form a gel matrix around the tissues. After gelling, the encapsulated plantlets were carefully removed and air-dried under sterile conditions to prepare for further growth. The encapsulated shoot tips exhibited faster development and higher regrowth rates compared to the nodal segments. (Figure 3) .

Table 1: Effect of ion exchange time on regrowth ability and average regrowth time of encapsulated explants of Mulberry (*Morus spp*.) variety kanava 2

Ion Exchange Time (Min)	Shoot Tips (%)	Average Regrowth Time (Days)	Nodal Segments (%)	Average Regrowth Time (Days)
10	43.2 + 2.7 b	15	36.7+ 2.3 b	21
20	92.5 + 0.3 a	10	82.3+ 0.6 a	20
30	27.4 + 2.0 b	17	21.2+ 2.1 b	27

The mean values are presented with their standard error (SEM). Within each column, distinct letters denote statistically significant differences in percentages, determined using Duncan's Multiple Range Test at a significance level of $p<0.05$.

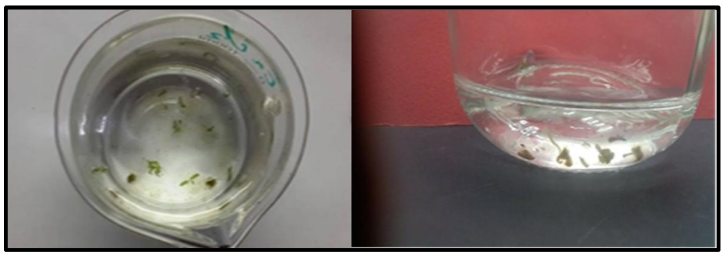

Fig. 2: The regenerated mulberry shoots were excised and immersed in the sodium alginate solution to ensure full coverage of the plant tissues.

Approximately 50 characteristic hydrogel beads were formed using 30 ml of sodium alginate and 100 ml of calcium chloride solution. The beads displayed variations in texture, shape, and transparency, depending on the sodium alginate and calcium chloride concentrations. Beads ranged in diameter from 3 to 5 mm. An encapsulation matrix of 3% sodium alginate and 50 mM calcium chloride was found to be the most effective for producing ideal beads. Lower sodium alginate concentrations (1–2%) resulted in poorly defined, soft beads unsuitable for handling, while higher concentrations produced isodiametric beads that were overly firm, delaying sprouting. Similarly, insufficient calcium chloride concentrations caused delays in gel matrix formation and negatively impacted bead quality.

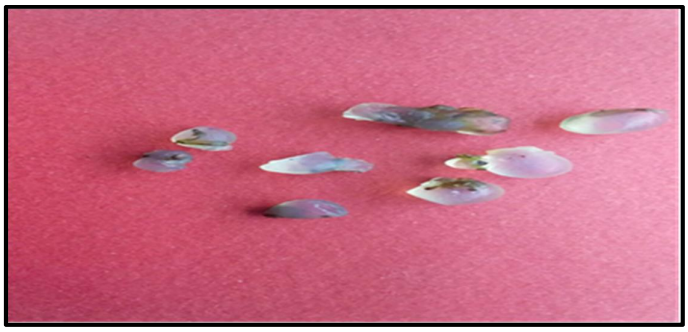

Fig. 3: After the gelled plantlets were carefully extracted, they were allowed to air-dry for several minutes under sterile conditions before being encapsulated in sodium alginate beads containing vegetative buds.

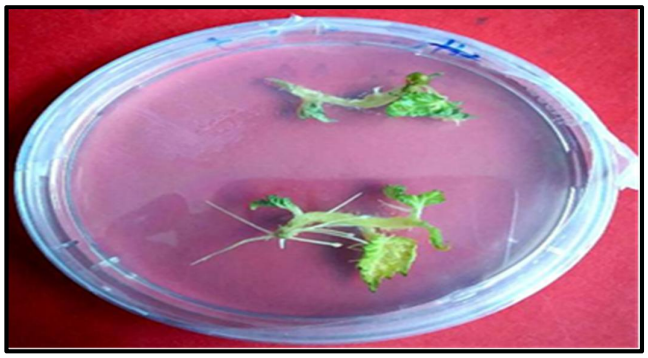

Fig. 4: The regeneration capacity and viability of Mulberry (*Morus spp*.) variety kanava 2

Regardless of the type of tissue or storage conditions, the explants demonstrated some degree of regrowth after 9 months of cold storage (Figure 5). At 3 months, there were no significant differences in regrowth between encapsulated and unencapsulated shoot tips or nodal explants. However, by 6 and 9 months, encapsulated shoot tips and nodal explants of *Morus spp.* (variety Kanava 2) showed significantly higher regrowth percentages compared to unencapsulated ones, under both light and dark storage conditions (Figure 5, B and C). Regrowth percentages for all explants under both growth conditions declined progressively as the storage duration increased (Figures 5 and 6), The decline in regrowth percentages was most noticeable between 3 and 6 months of storage. At both 6 and 9 months,

shoot tip explants exhibited a higher regrowth capacity compared to nodal explants. (Figure 3). The regrowth percentages of all explants in both growing conditions decreased over the storage period. (Figures 2 and 3), The decline in regrowth percentages of all explants was most noticeable between the 3- and 6-month storage periods. However, at 6 and 9 months, shoot tip explants exhibited a higher regrowth capacity compared to nodal explants (Figure 6).

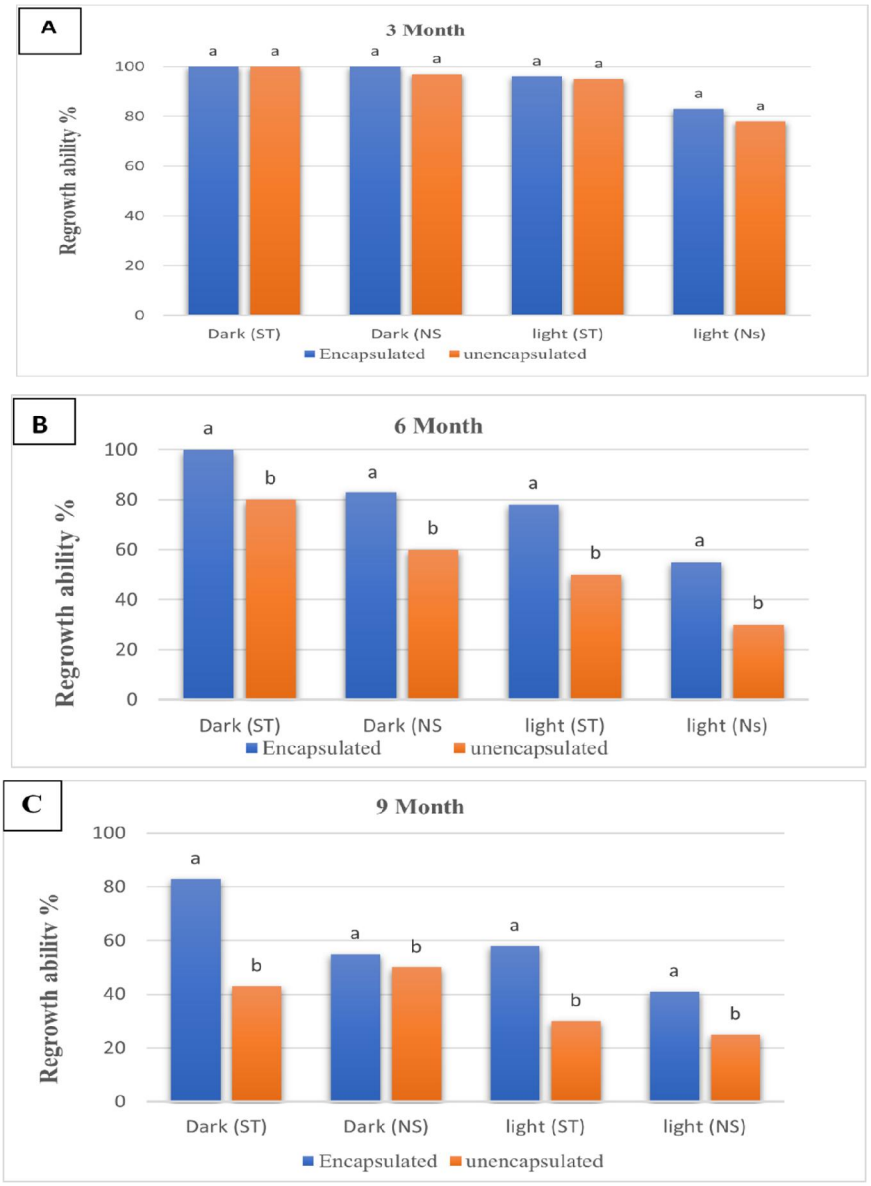

Fig. 5: The regrowth ability (%) of encapsulated and unencapsulated shoot tips (ST) and nodal segments (NS) of the Mulberry (*Morus spp*.) variety kanava 2 was evaluated after storage at 4°C under both dark and light conditions for three storage durations: (A) three Months, (B) six Months, and (C) nine Months. Significant differences in regrowth percentages between encapsulated and unencapsulated explants, as well as between dark and light storage conditions, were determined using the χ^2 test at $p \leq 0.05p$. Different letters denote significant differences within tissue types and storage conditions for each period.

Furthermore, the highest regrowth ability at 6 and 9 months was recorded for the encapsulated shoot tips kept in the dark (Figure 5). The highest regrowth capacity at both 6 and 9 months was observed in the encapsulated shoot tips stored in the dark (Figure 7). The preservation conditions evaluated were

more effective for the encapsulated shoot tips, with darkness enhancing regrowth at each storage interval. Overall, storage in the dark positively impacted the regrowth of the explants during all periods assessed (Figure 7). he preservation conditions tested were found to be more effective for encapsulated shoot tips compared to encapsulated nodal segments. No morphological changes were noted in the encapsulated explants that successfully developed into shoots. No changes in morphology were detected in the encapsulated explants that successfully developed into shoots.

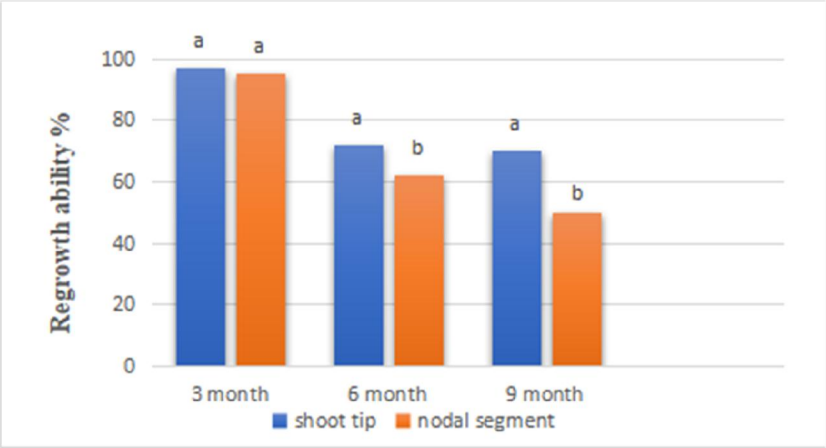

Fig. 6: The ability of encapsulated shoot tips and nodal segments to regenerate was compared after 3, 6, and 9 months of storage at 4°C. Significant differences in regeneration percentages between shoot tips and nodal segments at each storage duration were identified, as indicated by distinct letters (χ^2 test, $p < 0.05$).

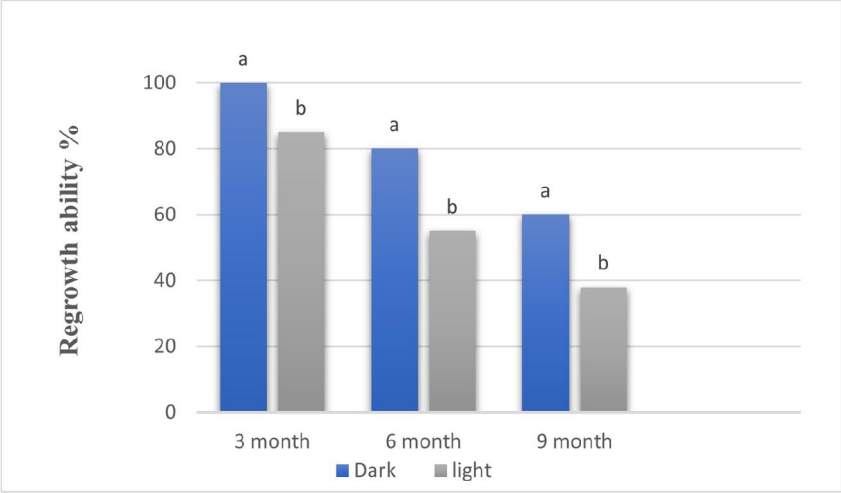

Fig. 7: The impact of light versus darkness on the regrowth of explants was evaluated after 3, 6, and 9 months of storage at 4°C. Significant differences in regrowth percentages between explants stored in dark and light conditions were identified at each storage period, as represented by distinct letters (χ^2 test, $p < 0.05$).

The chlorophyll content in mulberry leaves (*Morus spp.*) Kanva-2 was assessed, table 2 showing the highest **c**hlorophyll a concentration at 1.24, while chlorophyll b recorded the lowest at 0.49. The total chlorophyll content for this variety was 1.73, with a chlorophyll a to b ratio of 0.08, indicating a notable dominance of chlorophyll a in the leaf composition.

Table2: Chlorophyll a, b and Total chlorophyll content in Mulberry leaf

Mulberry variety	Chlorophyll			
	a	**b**	**Total**	**a/b**
***Morus spp.* (variety Kanava 2)**	1.24+0.53	0.49+0.28	1.73+0.17	0.08+0.05

4. Discussion

The characteristics of encapsulated explants are influenced by the concentrations of sodium alginate and calcium chloride, along with the duration of ion exchange, all of which play a crucial role in achieving optimal bead formation (Rai *et al.,* 2009). Sodium alginate paired with calcium salts has been identified as an ideal combination for encapsulation due to its affordability, gentle handling of tissues, ease of application, and high efficiency in converting propagules into viable plants (Sharma *et al.,* 2013). The firmness of the encapsulated beads largely depends on the extent of sodium ion exchange with calcium ions. To achieve consistently spherical and firm beads, the ion exchange duration needs to be carefully optimized, typically ranging from 20 to 40 minutes. (Standardi *et al.,* 2012). For Mulberry (*Morus spp*.) variety Kanava 2, a hardening duration of 20 minutes produced the most effective results, achieving 92% regrowth for shoot tips and 83% for nodal segments. While these findings highlight the specific efficiency for this variety, research on fruits, ornamentals, and vegetable species commonly indicates that a 30-minute ion exchange period is optimal for forming uniformly spherical beads with good sprouting potential. (Piccioni *et al.,* 1995, Sicurani *et al.* 2001 and NorAsmah *et al.,* 2011). For the encapsulated Mulberry (*Morus spp*.) variety Kanava 2, shoot tips demonstrated the shortest average regrowth time of 10 days when hardened for 20 minutes, while nodal segments required 20 days under the same conditions. Regrowth times can differ across species, for instance, using a similar encapsulation protocol, *Photinia fraserii* exhibited regrowth within 10 days. (Ozden-Tokatli *et al.,* 2008), In contrast, regrowth for *Nerium oleander* and *Kalanchoe spp***.** required 18 and 19 days, respectively, under similar encapsulation conditions. (Lambardi *et al.,* 2006). The ability of both encapsulated and unencapsulated explants to form shoots was influenced by the duration and conditions of storage. Encapsulated explants of Mulberry (*Morus spp*.) variety Kanava 2, maintained under slow-growth *in vitro* conditions, demonstrated superior adaptation to storage compared to their unencapsulated counterparts. This enhanced regrowth is likely due to the encapsulating gel matrix, which acts as an "artificial endosperm," providing a protective barrier and essential nutrients. These findings align with earlier research, which highlighted that the sodium alginate matrix not only shields the explants but also supplies nutrients, resulting in higher regrowth rates for encapsulated nodal segments relative to unencapsulated ones (Singh *et al.,* 2010). Encapsulation technology is a valuable tool for conserving plant material, particularly for medium- to long-term preservation. It has also been successfully applied in cryopreservation for various species, offering a practical method to maintain genetic resources over extended periods. (Gonzalez-Arnao *et al.,* 2006 and Padrò *et al.,* 2012). Several studies have highlighted the effectiveness of encapsulation techniques for short- and medium-term conservation, ranging from 1 month to 1 year, while maintaining the viability of the explants. This approach ensures the preservation of plant material over these periods without significant loss in quality or regeneration potential. (Hung *et al.,* 2011 and Srivastava *et al.,* 2009) lso help extend the time between subcultures by slowing down plant growth, effectively reducing the need for frequent transfers. This method is particularly useful for maintaining explant viability over extended periods, allowing for longer intervals between subcultures without compromising plant health or regeneration potential. (Tangamani and Vevekandan (1984) Under tropical conditions, higher chlorophyll content was observed in both MR2 and Japanese mulberry genotypes, with particularly elevated levels of chlorophyll 'a' and 'b.' These pigments play a crucial role in photosynthesis, enhancing plant growth and productivity. The higher chlorophyll content in these genotypes is beneficial for better photosynthetic efficiency. This also emphasizes the potential of encapsulation technology in advancing sustainable agricultural practices and supporting research in mulberry biotechnology, as it can optimize growth conditions and improve crop resilience. (Singh *et al.,* 2012; Ghosh *et al.,* 2020).

4. Conclusion

Encapsulation technology plays a pivotal role in the conservation and propagation of mulberry plants by addressing key challenges in genetic resource management and large-scale cultivation. It offers an efficient means for genetic preservation, allowing the storage of mulberry germplasm without frequent cultivation, thereby safeguarding valuable genetic material for future breeding and research. Additionally, it facilitates efficient transport, as encapsulated materials demonstrate resilience to environmental variations, ensuring the safe transfer of genetic resources over long distances without loss of viability. Furthermore, this technology supports mass propagation, enabling large-scale multiplication of mulberry plants while maintaining genetic uniformity and stability, which is crucial for consistent agricultural and commercial applications.

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