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## Optimization of Genetic Transformation System in *Brassica napus* via *Agrobacterium*

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### ABSTRACT

Efficient callus induction and plant regeneration are critical steps in plant tissue culture and genetic transformation. This study focused on optimizing nutrient media, plant growth regulators (PGRs), sterilization protocols, and transformation parameters to enhance regeneration efficiency in *Brassica* species. Murashige and Skoog (MS) medium supplemented with specific concentrations of cytokinins (BAP, KN) and auxins (NAA, IAA) proved most effective for callus induction and shoot regeneration. The optimal combination of 5.0  $\mu$ M BA and 0.5  $\mu$ M IAA yielded the highest regeneration rate (~90%), while Treatments with 5.0  $\mu$ M BA and 0.5  $\mu$ M IAA showed reduced efficiencies at 70% and 75%, respectively. Sodium hypochlorite (NaOCl) was the most effective surface sterilant, with eight rinsing cycles post-treatment leading to maximal regeneration by minimizing residual toxicity. Explant type significantly influenced outcomes; cotyledonary nodes and immature embryos demonstrated superior regenerative potential compared to leaf or hypocotyl tissues. Environmental conditions such as a temperature range of 25–28°C and controlled light intensity further improved culture performance. Sucrose at 2–3% served as the optimal carbon source, balancing energy supply and osmotic pressure. These findings support an integrated protocol for efficient genetic transformation in *Brassica*, facilitating future applications in functional genomics and crop improvement.

**KEYWORDS:** Brassica, Rapeseed, Genetic transformation, *Agrobacterium*, CTAB

### INTRODUCTION

Rapeseed (*Brassica napus*), generally known as oilseed rape. Globally it is one of the crucial high oil content crops, widely used for human consumption, animal feed and biofuel production. Besides its economic importance, its cultivation faced various substantial challenges including biotic and abiotic stresses. In recent years, rapeseed has become an essential agricultural commodity contributing to global food security and sustainable energy production (Almasi, 2020). The quality and yield of rapeseed crop were adversely affected by number of insect pests, various diseases caused by fungus, drought and salinity stresses as well. In this regard, the most impressive plant biotechnological approaches especially genetic modification and tissue culture techniques have shown great prominence considerably for improving the resilience and productivity of *Brassica napus* (Chalhoub *et al.*, 2014).

*Agrobacterium*-mediated transformation method is one of the most widely used techniques for genetic improvement in rapeseed. However, development of genetically modified rapeseed varieties through the efficiency of this transformation process remains major obstacles. The success of transformation and subsequent plant regeneration play a crucial role in determining the factors such as plant genotype, explant type and culture conditions (Oliveira & Yu, 2022; (Gelvin, 2003). Inhibition of root and shoot growth and

development during the co-cultivation with *Agrobacterium*, the process is being complicated by issues such as tissue necrosis, oxidative stress, which ultimately delayed the callus formation (Hassan *et al.*, 2021). Result for low shoot and root formation in some cultivars exhibits the genotype dependent regeneration potential in *B. napus* unlike model plants such as *Arabidopsis thaliana* (Hu *et al.*, 2019).

By enhancing and optimizing the different parameters for infiltration, the most effective and efficient method of *Agrobacterium*-mediated transformation in *Brassica napus* was successfully established. The gene functional studies related to an efficient and stable genetic transformation system for brassica have limitations. Therefore, by modifying their levels of improving the transformation success rate in some *B. napus* cultivars, the most researchers initially promoted callus initiation and organogenesis (Liu *et al.*, 2018). Of these, one such approach involves modifying the nutrient culture medium, specifically by regulating the concentration of plant growth regulators (PGRs) such as cytokinin and auxins (Matthaus *et al.*, 2016). These PGRs play a significant role in manipulating cell regeneration and differentiation. Additionally, the temperature has proven to be another effective strategy to enhance transformation efficiency and optimize the cultivation conditions such as the duration of co-cultivation (Liu *et al.*, 2012). Thus, improving crop yield and sustainability, these

promoters when inoculated with *Agrobacterium*-mediated transformation, have the potential to produce rapeseed plants better equipped to withstand environmental stresses and adequate yield (Qian *et al.*, 2018).

## MATERIALS AND METHODS

### Preparation of explant material for genetic transformation

**Collection of explant material:** The explants (seeds) of rapeseed were collected from the marketplace of Hyderabad district and brought into the Plant Molecular and Plant Tissue Culture Laboratory, Department of Biotechnology for further experimentation. Initially, seeds were washed with sterilized distilled water and then subjected to sterilization process. The washed seeds were surface sterilized in order to remove the possible microbial contaminants.

**Soaking of collected seeds:** Potency healthy seeds of rapeseed were soaked in distilled water for 24 hours to initiate the germination. After that, the soaked seeds were tapped carefully and rinsed optionally thrice to remove the substances that could stall growth. Further, seeds were prepared for culturing and initially spread on paper to remove excess content of moisture.

**Preparation of MS nutrient media:** Ingredients of (MS) basal media, contained the macro, microelements and vitamins were carefully measured and transferred approximately 800 mL of distilled water in a sterilized beaker. For making readymade MS media, about 4.4 grams of the MS powder was measured and transferred to beaker for dissolving. Once the MS powder was fully dissolved, the pH of the medium was adjusted to 5.8-5.9. After reaching the anticipated pH reading, the total volume up to 1 liter was adjusted by adding more distilled water. After that, media was autoclaved at 121°C for 20min for tissue culture.

**Surface sterilization:** Effective sterilization is important for tissue culture and preparing the seeds for culturing as well. Initially seeds were washed with sterilized distilled water followed by treated with 70% ethanol about 1 min and 40%(NaOCl) solution for 20 min, the solutions were suspended with a few drops of Tween-20. After that, the seeds were thoroughly washed in about 6 times with sterilized distilled water. All these processes are being done under the laminar airflow cabinet.

**Tissue culturing of rapeseed:** Culturing was done according to the method reported by scientists. Explants were cultured on each of basic medium supplemented with various continuous concentrations of growth hormones at different seedling regenerated stages. In addition, the regenerated shoots were separated and relocated to rooting medium. Under aseptic conditions in the laminar airflow cabinet, the sterilized seeds were transferred onto the prepared MS medium, placing them flat for germination or cutting them open for callus induction.

**Extraction of DNA from regenerated seedlings of rapeseed:** To extract DNA from the leaves of regenerated rapeseed seedlings, approximately 100 mg of fresh green leaf tissue was collected and immediately frozen at 0°C. In short, 10ml of 2 X (CTAB) solutions were preheated under water bath condition at 65-70°C. The mortar and pestle were pre-cooled at -80°C. Then about 5g of leaves were frozen in liquid nitrogen. Afterwards, the crushed powder was transferred to the preheated 50ml solution in a tube. The tube containing 10ml 2XCTAB solution was then centrifuged at 5000rpm for 20 minutes. Further process was followed with only few modifications as described. In addition, the CIA liquid was added and incubated at 37°C, and then centrifuged at 3000rpm on room temperature for about 20 minutes, transferred the supernatant to a new 1ml plastic tube. Slowly added precipitation buffer and gently shaken for 15-20 times until the DNA precipitates together. Afterward, the precipitate was washed with ethanol solutions: 70% ethanol for 7 minutes; 100% ethanol for 5 minutes. Finally, it was stored at 0°C for further experimentation.

**Gel Electrophoresis and Documentation:** For the process of gel electrophoresis, initially 1% gel was prepared. Once the gel was solidified approximately 15µL of total extracted DNA sample was mixed and 1µL of ethidium bromide (EB) and 1 µL of loading buffer was added. This mixture was carefully loaded into the wells of the gel. A 1% or 1.5% agarose gel was prepared by dissolving agarose powder in a buffer solution, depending on the desired resolution for the DNA fragments. After running the gel was visualized under UV light using a gel documentation system.

**Quantification of DNA through Spectrophotometry:** The absorbance values obtained were directly proportional to the concentration of DNA in the sample, allowing for accurate quantification. In this process, the DNA sample was measured at a wavelength of 260 nm, where nucleic acids absorb UV light. Good quality DNA samples characterized by a high A260/A280 ratio indicate minimal contamination with proteins or other impurities.

**Histological analysis of cells using microscopy:** Leaf samples were collected 0.5-cm by 30.4-cm slices from various regions. Measurement of cell size and quantification of cell numbers was performed as described by Ma *et al.* (2008). For microscopy, slices of middle region representing 50% of the leaf length were selected. These slices were then fixed in formaldehyde. Further process was performed as a reported standard protocol. Scanning microscopy was performed using a Philip (S3400N) apparatus. The AbsE cell death photography and total cells count were executed. Afterward, the total number of AbsE cells were counted in the field of view using ImageJ software.

**Preparation of Agrobacterium for gene transformation:** In order to prepare the *Agrobacterium tumefaciens* strain for successful gene

transformation, a single colony of *Agrobacterium* harboring the desired DNA molecule was inoculated into liquid Luria Bertani (LB) medium composited with the appropriate antibiotics to ensure selective growth. This incubation period allowed the bacteria to proliferate until they reached the log phase of growth, indicated by an optical density (OD<sub>600</sub>) of approximately 0.6 to 1.0. The culture was incubated overnight at 28°C with shaking at 200-250 rpm. The resulting pellet was then resuspended in a sterile infiltration buffer composed of 10 mM magnesium chloride (MgCl<sub>2</sub>), 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), and 100 µM acetosyringone.

## RESULTS

**Screening of Basal Media for Callus Induction and Regeneration:** Each medium has different nutrient compositions that can significantly influence the growth and development of callus and subsequent plant regeneration (Figure 1). MS medium is rich in nutrients, making it highly effective for many plant species. The optimize regeneration efficiency and callus induction proficiency, the research combined the MS basal media with different composition and concentration of plant growth regulators (PGRs). For instance, adding casein hydrolysate (CH) and coconut water (CW) to the basal medium enhances callus growth and organogenesis. Different concentrations of PGRs like KN, BAP and NAA were tested to find the optimal combination for specific plant species.

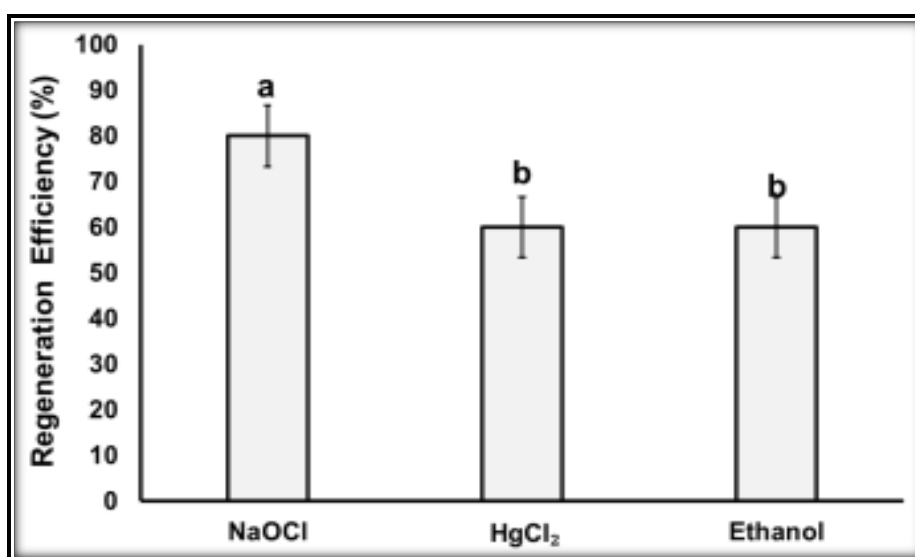


**Figure 1.** Effect of different sterilizing agents used for sterilization of explant source before tissue culturing

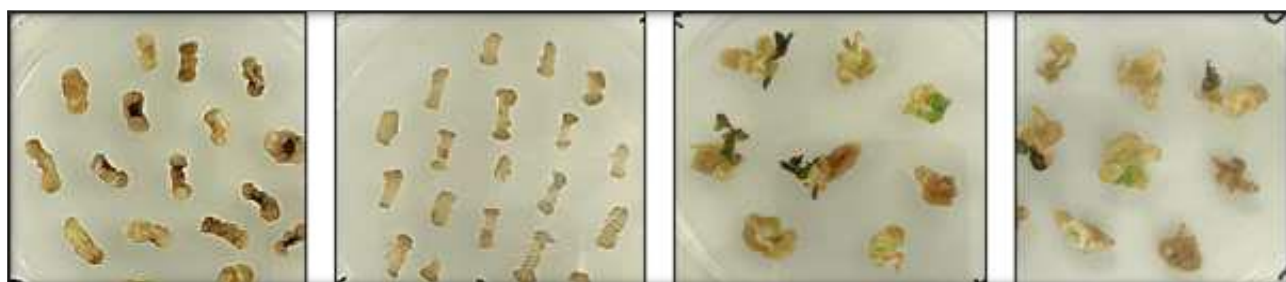


**Figure 2.** Initiation of shoot regeneration from induced callus for *Agrobacterium* transformation

**Optimization of plant growth regulators for enhanced shoot Formation:** In present study, to optimize the shoot, research typically experimented with different combinations and concentrations of cytokinin's (Figure 2). For shoot multiplication, a combination of BA and IAA was used with optimal results observed at concentrations such as 5.0 µM BA and 0.5 µM IAA. The Control treatment achieved the

highest regeneration percentage, approximately 90%, serving as a baseline for comparison (Figure 3). Treatment 1 displayed a notable reduction in regeneration, with a percentage of about 70%, reflecting a 22% decrease compared to the Control (Figure 4). Further detailed regulation on the specific concentration and requirement would be required to explore the controlled regulatory mechanisms.

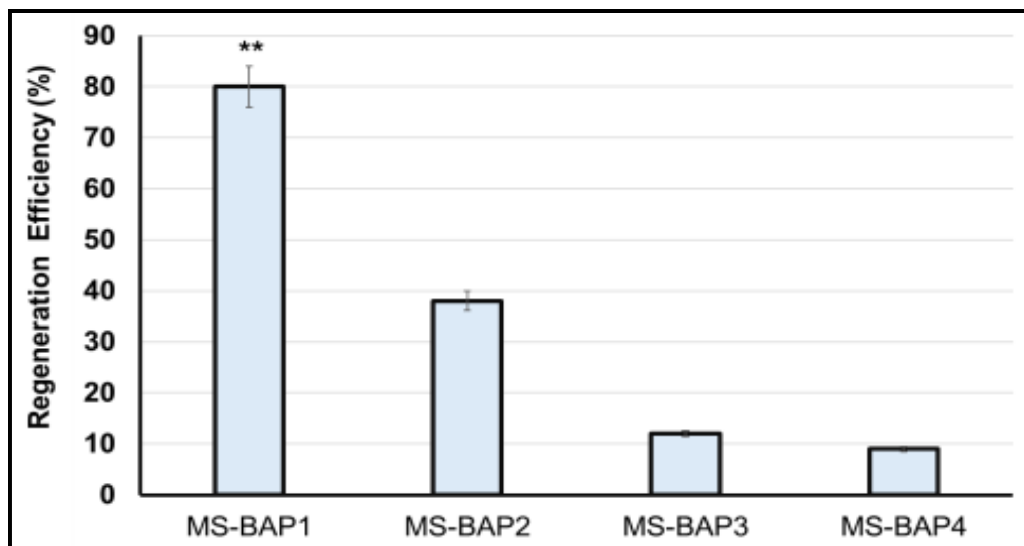


Figure 3. Effect of different growth hormones for optimizing shoot regeneration efficiency

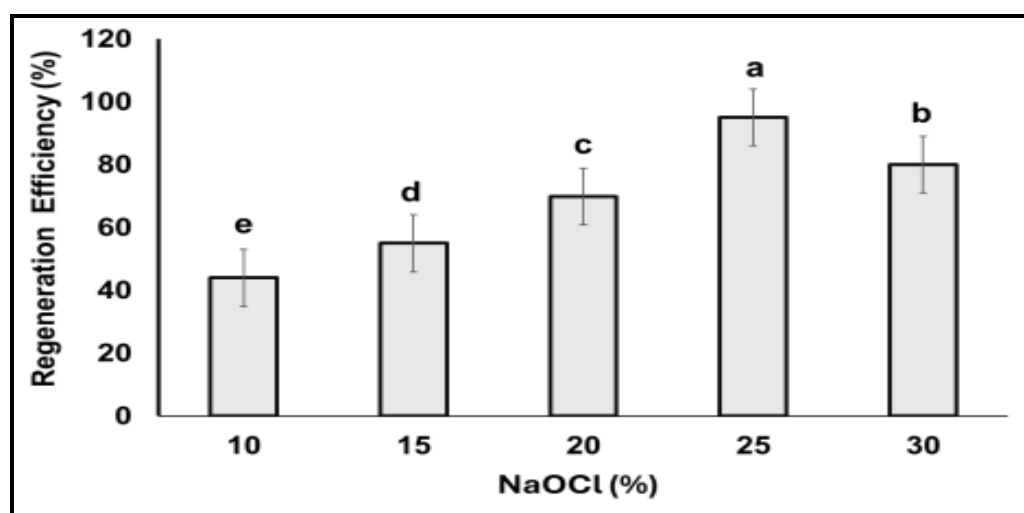


Figure 4. Effect of different percentage of NaOCl for surface sterilization of explant

**Effect of explant type on regeneration efficiency:** In present study, different seeds, whole leaves and the basal parts of leaves were used and found more competent for shoot regeneration compared to the distal parts. Cotyledonary nodes are particularly effective for direct shoot regeneration, often showing higher regeneration rates compared to other explants like cotyledons, hypocotyls, and leaves. Immature

embryos were widely used for cereal crops due to their high regenerative potential (Figure 5). Therefore, the choice of explant type used to influence the plant species, growth stage, and specific requirements for regeneration. Leaf explants are often preferred for shoot regeneration, although their effectiveness may be affected by factors such as leaf integrity and orientation.

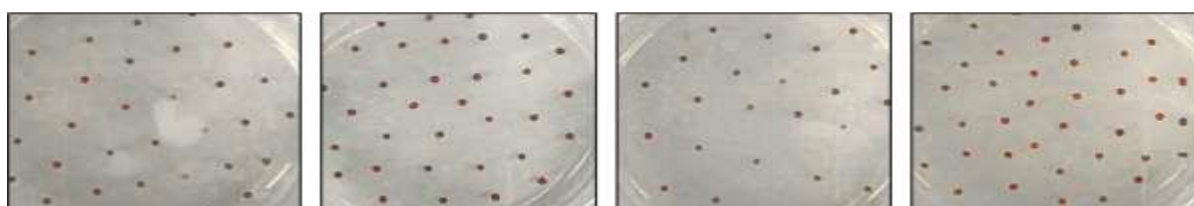


Figure 5. Sterilized seeds used for embryogenesis as explant type on regeneration efficiency

**Impact of temperature fluctuation and light intensity on optimal tissue culture:** Temperature is a critical environmental factor in plant tissue culture, significantly influencing growth, differentiation, and

overall success of the culture. The results highlight how different temperature levels affect the growth and regeneration potential of plant tissue cultures (Figure 6). Tissues in adverse temperature range often exhibit reduced callus formation, poor shoot regeneration, and

sometimes tissue browning, possibly due to stress-induced damage. Intermediate temperatures (25–28°C) produce the highest regeneration and growth rates,

indicating the optimal range for most plant tissue cultures.

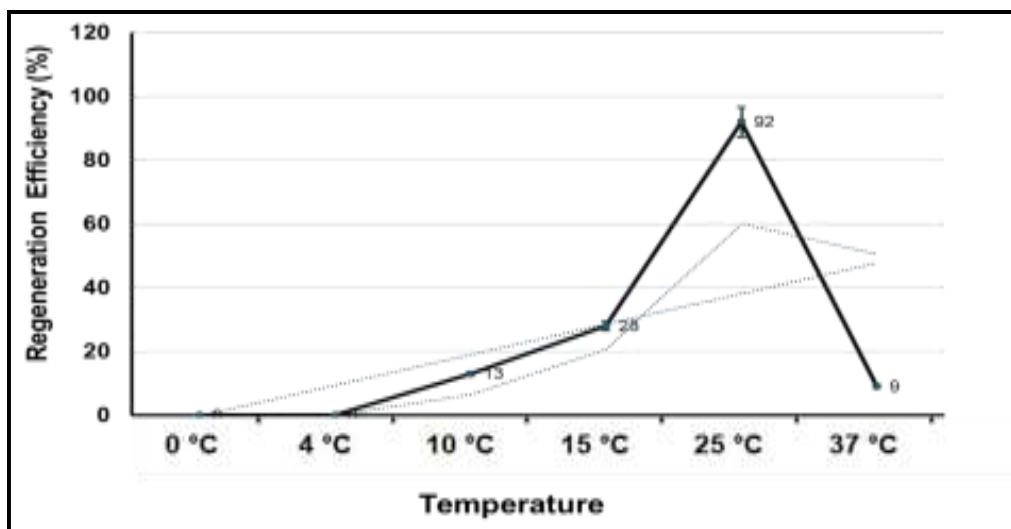


**Figure. 6.** Effect of different degrees of temperature on regeneration efficiency of explants

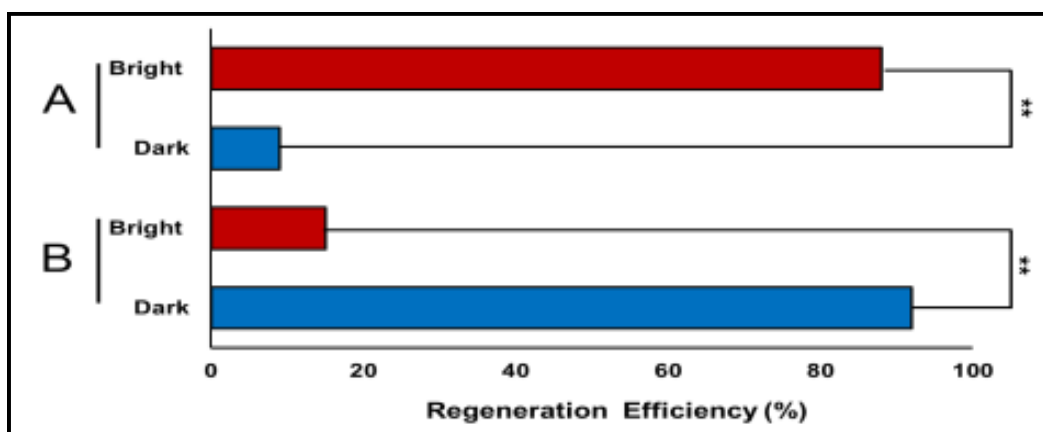


**Figure. 7.** Effect of light intensity on regeneration efficiency of explants

**Influence of carbon sources on tissue growth:** Carbon sources are essential in plant tissue culture as a source of energy and serve as a carbon basis for various metabolic processes. Osmotic regulators, such as sugars and sugar alcohols, help maintain the osmotic potential of the culture medium, which is crucial for cell growth and differentiation. The concentration of the carbon source significantly affects tissue growth. Increasing the concentration of sugars generally enhances growth until an optimal level is reached, beyond which it may inhibit growth due to osmotic stress. For instance, sucrose concentrations between 2% to 3% are commonly used for optimal growth in many plant species

**Quantification of DNA from the leaves of regenerated seedlings:** To further assess the quality of

the extracted DNA, agarose gel electrophoresis was performed. The gel analysis showed that most of the DNA was intact, appearing as a clear, high molecular weight band with minimal degradation. In a few samples, faint bands were observed, which may have been due to partial degradation, possibly from the age of the leaf tissue or handling during extraction. The successful amplification of the transgene from the DNA of regenerated seedlings indicates that the *Agrobacterium*-mediated transformation process was efficient, resulting in the stable integration of the transgene into the plant genome.

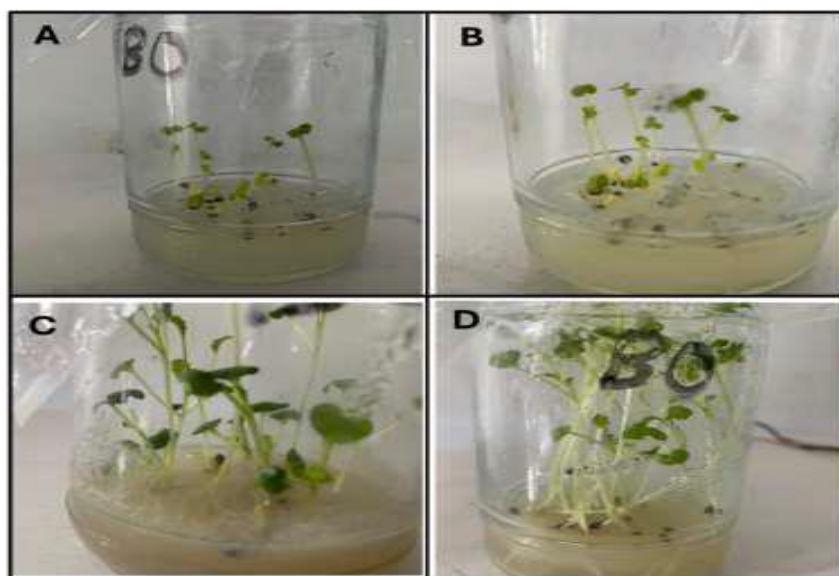


**Figure 8.** Inoculation on sterilized seeds on optimized nutrient media for regeneration of shoots used for *Agrobacterium* infestation

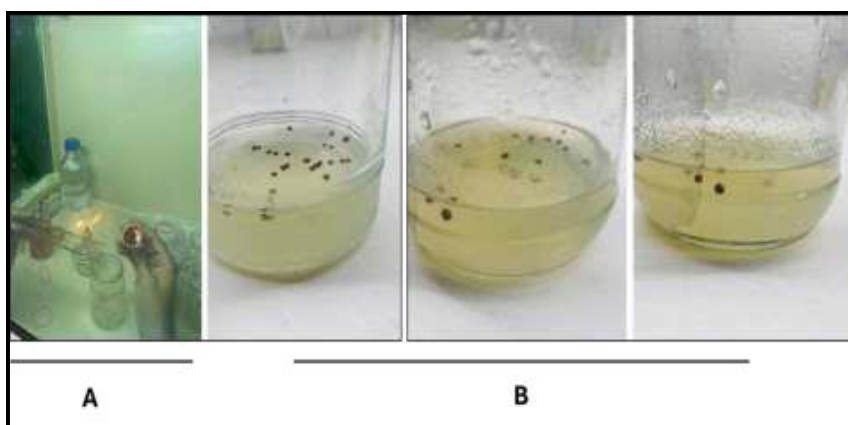


**Figure 9.** Seedlings regenerated optimized nutrient media for DNA extraction and further molecular studies

**Table 1** Quantification DNA through spectrophotometry. DNA concentration determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer using a quartz cuvette

| Sample    | Concentration (ng/ul) | A260/A280 | 260/A230 |
|-----------|-----------------------|-----------|----------|
| Sample. 1 | 92.7                  | 1.89      | 2.30     |
| Sample. 2 | 76.5                  | 1.75      | 1.95     |
| Sample. 3 | 101.3                 | 1.91      | 2.25     |
| Sample. 4 | 88.0                  | 1.87      | 2.15     |
| Sample. 5 | 95.6                  | 1.84      | 2.05     |

#### **Agrobacterium concentration and infection Time:**

The concentration of *Agrobacterium* (*Agrobacterium tumefaciens*) was found to significantly impact transformation efficiency. A bacterial suspension concentration of  $OD_{600} = 0.6$  was identified as optimal for the transformation process, as it yielded the highest transformation rate without overloading the explant tissue with excessive bacterial cells, which could lead to tissue damage. Infections carried out for 30 minutes resulted in the highest number of transformed cells. Shorter infection times (e.g., 15 minutes) resulted in significantly lower transformation efficiencies, likely due to inadequate bacterial attachment, while prolonged infection times (e.g., 60 minutes) led to a

decrease in regeneration potential, possibly due to excessive bacterial overgrowth.

**Explant type and surface area:** To further investigate the usage of explant and its surface area, the leaf explants, especially those from seedlings, showed the highest transformation efficiency compared to hypocotyl or cotyledon explants (Figure 8). Their larger surface area facilitates better attachment of *Agrobacterium* and improves shoot regeneration after transformation (Figure 9). Additionally, cutting explants into smaller pieces may further enhance regenerative capacity by increasing the number of explants available for transformation. This expanded surface area promotes stronger *Agrobacterium*

interactions, thereby increasing transformation success.

**Co-Cultivation Conditions:** Co-culture conditions play a crucial role in maximizing transformation efficiency. Co-cultivation in solid media under dark conditions for 2-3 days yields the highest transformation rates. During this time, T-DNA can be efficiently transferred from *Agrobacterium* to plant cells, while the absence of light reduces explant stress, supporting optimal growth and regeneration.

**Selection and regeneration media:** The presence of cytokinins such as BAP (6-benzylaminopurine) in the

regeneration medium promoted shoot formation, with the highest regeneration observed at 1.0 mg/L BAP. Additionally, the combination of BAP with IAA (indole-3-acetic acid) helped promote rooting, further enhancing plant recovery and survival. The explants were transferred to a selective regeneration medium containing kanamycin to select for transformed cells (Figure 10B). The optimal concentration of kanamycin for Brassica was found to be 50 mg/L, as higher concentrations resulted in poor shoot regeneration due to toxicity, while lower concentrations allowed non-transformed cells to proliferate (Figure 10A).

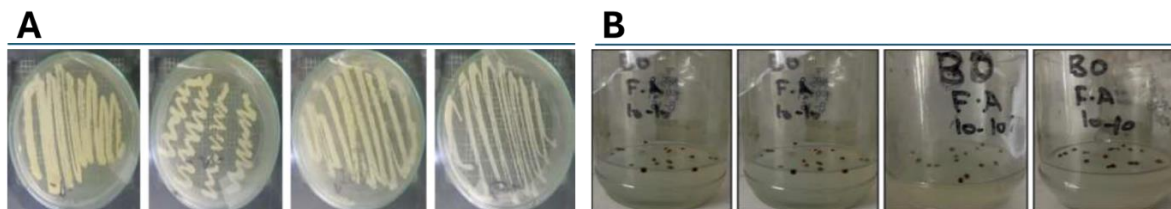


**Figure 10. A) Growing of *Agrobacterium* for transformation. B) Emerging embryos subjected to *Agrobacterium* for transformation.** Emerged seeds were dipped in *Agrobacterium* solution and then grow on nutrient medium containing kanamycin antibiotic

## DISCUSSION

This study focused on the preparation of genetic material to improve *Agrobacterium*-mediated transformation in *Brassica napus*. By ensuring the integrity of genetic material and analyzing key transformation elements computationally, this study contributes to improving the efficiency and precision of transgene integration in rapeseed. In this study, after establishing the high efficiency *Agrobacterium*-mediated genetic transformation system, we explore that the inducible expression in different brassica genes promotes callus formation and induced shoot regeneration. This research provides a technological foundation to gain insight into gene function in rapeseed in future studies and achieves the breakthroughs in the genetic transformation in rapeseed. However, there are always techniques and trends that can be used depending on the researcher and the lab conditions (Mekso and Mena, 2022).

The preparation of high-quality genetic material is a critical step in *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformation is widely used for gene delivery in plants (Chalhoub et al., 2014). In this study, DNA was extracted from the leaves of regenerated *Brassica* seedlings to quantify the success of *Agrobacterium*-mediated transformation and confirm the presence of the transgene. The A260/A280 ratio, which is used to assess DNA purity, ranged from 1.8 to 2.0 in most samples, indicating that the DNA was free from significant contamination by proteins or phenolic compounds. The agarose gel electrophoresis was performed to further assess the quality of the extracted DNA bands with minimal degradation having high molecular weight were analyzed and showed that most of the DNA content was intact and appeared as a transparent. The age of the leaf tissue and handling

method during extraction restricted few samples and the faint bands were observed. These issues found which may have been due to the reason of partial degradation (Hassan et al., 2021). The functional genomics applications demonstrate the successful genetic modification of *Brassica* for validate the transformation protocol. Reduce the risk of genetic transformation and degradation shown that high purity plasmids contribute to increased transformation efficiency (Chen et al., 2023).

Optimization of Codon has shown to significantly enhance the gene expression in transgenic plants for vectors transformation including sequences of gene containing high level of promoters (Liu et al., 2023). In addition, gene transfer during *Agrobacterium*-mediated transformation ensuring the structural accuracy and evaluation of the T-DNA. Research shows that gene rearrangements and improve the transformation success and maintaining the integrity of the T-DNA structure that likely reduced. Recent advances have focused on modifying *Agrobacterium* strains to enhance virulence and improve DNA transfer efficiency (Kumar et al., 2023). Considering the sensitivity of explant to the *Agrobacterium*, the final concentration was found to be easily controlled by the volume of the original *Agrobacterium* solution rather than an adjustment to avoid explants turning necrotic. Therefore, ensuring the appropriate bacterial activity of collected *Agrobacterium* was of great significance. Moreover, the timing of incubation infection and co-cultivation stage should be carefully controlled, as excessive attachment can directly lead to explant necrosis or reduced regeneration. Usually, 10 min was used for hypocotyls infection, with maintenance for 36–48 h in the co-cultivation medium in this investigation. After 24 h of co-cultivation in co-cultivation medium, the hypocotyls should be tough

and bright white, which can be transferred to CIM 24 h later.

If the hypocotyls do not show the features, it was suggested that they should be transferred to the CIM immediately or up to 12 h later to avoid explant necrosis (Khan *et al.*, 2022). In this way, highly efficient transformation was obtained in eight rapeseed varieties. In conclusion, this study successfully prepared and analyzed genetic material for transformation of *Brassica napus* to optimize transformation components. These results reinforce the critical role of bioinformatics in improving transformation efficiency by ensuring the integrity of genetic elements and minimizing unintended genetic alterations. While *Agrobacterium* -mediated transformation remains a powerful tool for genetic modification.

## CONCLUSION

This study successfully optimized the genetic transformation system in brassica via *agrobacterium* by fine tuning the key factors such as nutrient media composition, PGR concentration, sterilization methods and culture conditions. The identified combination of 5.0µM BAP and 0.5µM of IAA along with effective sterilization and optimal explant selection, significantly enhanced genetic transformation efficiency. These results provide a healthy groundwork for future genetic transformation and functional genomics studies aimed at improving Brassica crop traits.

## AUTHORS' CONTRIBUTION

FI performed all the experiments and prepared the initial draft. AJK conceptualized the study, supervised the work, and contributed to writing the original draft. MA and SA revised the manuscript and validated the data. AG and FAM conducted the formal analysis and edited the figures

## CONFLICT OF INTEREST

The manuscript has been reviewed by all authors, and it has been confirmed that no conflicts of interest exist.

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