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Enhancing Agrobacterium-Mediated Cotton Gene Transformation by Optimizing Co-Cultivation Conditions and Inoculum Density

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Abstract

Background: Cotton (*Gossypium hirsutum* L.) is a major commercial crop, but its genetic transformation remains challenging due to recalcitrance, high phenolic synthesis, and low regeneration efficiency. **Objective:** This study optimized critical parameters specifically bacterial culture density (OD600), co-cultivation duration, and plant growth regulator combinations to enhance the efficiency of Agrobacterium-mediated transformation and tissue regeneration in cotton. **Materials and Methods:** Hypocotyl segments were infected for 10 minutes at various optical densities (OD600) and co-cultivated on Murashige and Skoog (MS) medium supplemented with 200 µM acetosyringone for different durations. Transformed tissues were selected on MS media containing 100 mg/L kanamycin following antibiotic sensitivity investigation and carrot disc testing of the Agrobacterium strain. **Results:** The highest transformation efficiency was achieved with an Agrobacterium density of (OD600) = 0.8 paired with a 3-day co-cultivation period. MS medium supplemented with 2,4-D (0.5 mg/L) and Kinetin (1.0 mg/L) produced the highest rate of callus induction whereas 2,4-D (1.0 mg/L) combined with BAP (0.5 mg/L) was optimal for callus proliferation. **Conclusion:** Optimizing bacterial density, co-cultivation period, and specific growth regulators significantly improves transformation efficiency and callus development in transgenic cotton.

Keywords: Cotton (*Gossypium hirsutum* L.), Agrobacterium-mediated gene transformation, Co-Cultivation, 2,4-D.

INTRODUCTION

Cotton is one of the world's most commercially significant crops. Cotton's long, cellulosic, single-celled fiber is perhaps what the general public is most familiar with, but other cotton products, like cottonseed, are also essential to the economy. Cottonseed is used to make oil, meal, linters, and hulls. Cottonseed is the second-most-produced oilseed in the world, after soybeans [1,2]. The food sector uses refined cottonseed oil, which is also the favored vegetable oil in several regions of the world [1]. While the hulls are added to the meal as roughage, the cottonseed meal left over after oil extraction can be utilized as a protein-rich feed for ruminants. One of the first commercially developed and widely accepted genetically modified species was transgenic cotton. Researchers have occasionally attempted to increase the quality of cotton crops, either by increasing the yield of fiber or by adding resistance to pests. However, cotton is considered a recalcitrant species for genetic transformation due to its tendency for high phenolic synthesis and low regeneration efficiency, making the process laborious and time-consuming.

The most popular technique for introducing genes into plants is Agrobacterium-mediated transformation. Typically, an explant, a small excised piece of plant tissue, is used for transformation. Tissue culture procedures are then used to restore the tiny amount of altered plant tissue into a full plant. Agrobacterium was first known to alter plants in 1983 [3]. Since then, significant progress has been achieved in expanding the range of plant species that Agrobacterium can transform and regenerate. The first genetically modified cotton plant was reported in 1987 [4,5].

Although Agrobacterium-mediated genetic transformation has produced stable transgenic cotton, more attempts have been made to improve the transformation procedure [6]. The Agrobacterium strain, the

kind and age of explants, the length of co-cultivation, the plant regeneration method, the selection marker, and the vector type are important factors affecting transformation success [4,7, 8]. The second most popular industrial raw material crop in Myanmar is cotton, which is crucial for the manufacture of apparel. However, bollworms cause cotton production to drop annually for cotton farmers. In an attempt to solve this issue, local farmers have been overusing pesticides, which can destroy beneficial insects, degrade soil and the ecosystem, and even endanger human health. Biotechnology has been used globally to create crops that are resistant to pests and illnesses without endangering human health or the environment. The main technique for creating transgenic cotton plants is Agrobacterium-mediated transformation. By exploiting the natural DNA transfer system of Agrobacterium tumefaciens, this technique allows for the stable integration of foreign genes into the cotton genome. Although transformation rates have increased significantly since the first reports of successful cotton transformation [4,5], further improvements are still necessary to enhance their efficiency. The type and physiological age of the explant, as well as the composition of the co-cultivation medium, are key factors influencing the optimal conditions for Agrobacterium concentration and co-cultivation. This study, therefore, investigates the effects of Agrobacterium concentration and co-cultivation duration on the transformation efficiency of cotton explants in an in vitro system.

MATERIALS AND METHODS

Preparation of Agrobacterium Culture

A single colony of *Agrobacterium tumefaciens* (with the gene of interest and selectable marker) is inoculated into a liquid YEP medium with 50mg/L Kanamycin. The culture is grown overnight on a shaker incubator at 28 °C until it reaches the late logarithmic growth phase (OD_{600} 1.0).

Preparation of Bacterial Suspension for Infection

The bacterial cells are pelleted by centrifugation and resuspended in a liquid co-cultivation medium, MS salt-based, supplemented with acetosyringone 200 µM. This step defines the 'Concentration' treatment variable. The optical density at 600 nm (OD_{600}) is adjusted to specific levels to create the Agrobacterium Concentration OD_{600} 0.4, 0.6, 0.8, and 1.0.

Inoculation and Co-cultivation Duration

Cotton seeds are surface sterilized and germinated in MS_0 medium. After two weeks, hypocotyl segments and cotyledon discs from the seedlings are immersed in the different bacterial suspensions for 10 min with gentle agitation. The inoculated explants are blotted dry on sterile filter paper and transferred onto solid co-cultivation medium with Kanamycin 100 mg/L. The plates are sealed and placed in the dark at a controlled temperature (27 °C) for varying periods (2, 3, or 4 days). After the co-cultivation period, the explants are washed with antibiotic (Carbenicillin 250 mg/L) and transferred to selection medium with various hormone combinations 2,4-D (0.5 mg/L) + Kinetin (1.0 mg/L), 2,4-D (1.0 mg/L) + Kinetin (1.0 mg/L), 2,4-D (0.1 mg/L) + Kinetin (0.5 mg/L), and 2,4-D (0.1 mg/L) + Kinetin (1.0 mg/L) for callus induction and proliferation.

Data Collection and Analysis

After two weeks, data were collected on: Explant Survival Rate (%): The percentage of explants that remain viable without necrosis, serving as an indicator of regeneration potential post-infection.

RESULTS

Although Agrobacterium-mediated genetic transformation has produced stable transgenic cotton, more attempts have been made to improve the transformation procedure [6]. However, the Agrobacterium concentration (OD_{600}) and the co-cultivation duration are highly interdependent and critical factors influencing the success of Agrobacterium-mediated transformation in cotton. Their optimization represents a delicate balance between maximizing T-DNA delivery and minimizing tissue damage. Therefore, in order to investigate the bacterial optical densities and length of co-cultivation in cotton transformation, Agrobacterium strains with Cry1Ab and EPSP gene were acquired from Creative Biogene Co., Ltd. (USA).

First, the strain was cultured in YEP medium and tested for antibiotic sensitivity using different concentrations (0, 50, 100, 150, 250 mg/L) of Carbenicillin. Among the concentrations, 250 mg/L of Carbenicillin effectively controlled the *Agrobacterium* strains (Figure 1). Moreover, the pathogenicity of the *Agrobacterium* strains harboring the Cry gene was confirmed using a carrot disc bioassay, where crown gall formation was observed after three weeks (Figure 2).

Bacterial optical densities were studied using OD_{600} values of 0.4, 0.6, 0.8, and 1.0 for transformation efficiency. When cotton explants were co-cultured at an OD_{600} of 1.0, it was found that the explants dried out and withered after two weeks. The optimal concentration of OD_{600} 0.8 was the best for cotton explant transformation. It kept bacterial overgrowth and tissue damage under control while providing a high enough bacterial density for effective T-DNA delivery to a large number of competent cells.

The efficiency of transformation can also be impacted by the length of co-cultivation. For 1, 2, 3, and 4 days, the co-cultivated explants in our study were kept in the dark. Co-cultivation periods of only 1 to 2 days limited bacterial proliferation and reduced the likelihood of tissue necrosis; however, this shorter duration provided insufficient time for the multi-step Agrobacterium-mediated transformation process. On the other hand, on the 4th day following infection, the cells at the cut edge of Agrobacterium-infected cotyledon disks exhibited maximum transient activity. This resulted in excessive bacterial growth that was difficult to control, even with subsequent antibiotic washes, and caused severe necrosis and death in the explants. The ideal duration for cotton transformation efficiency was found to be 3 days when the explants were kept in the dark. It struck a compromise between providing adequate time for stable integration in a sizable number of cells and minimizing the detrimental effects of prolonged bacterial co-culture (Table 1).

The co-cultivated explants were cultured and propagated in selection medium with various hormone combinations: 2,4-D (0.5 mg/L) + Kinetin (1.0 mg/L), 2,4-D (1.0 mg/L) + Kinetin (1.0 mg/L), 2,4-D (0.1 mg/L) + Kinetin (0.5 mg/L), and 2,4-D (0.1 mg/L) + Kinetin (1.0 mg/L) for callus induction. It was found that the combination of 2,4-D (0.5 mg/L) + Kinetin (1.0 mg/L) was the best callus induction medium. Subsequent tests to proliferate the obtained callus revealed that the medium containing 2,4-D (1.0 mg/L) + BAP (0.5 mg/L) was the most effective for callus proliferation.

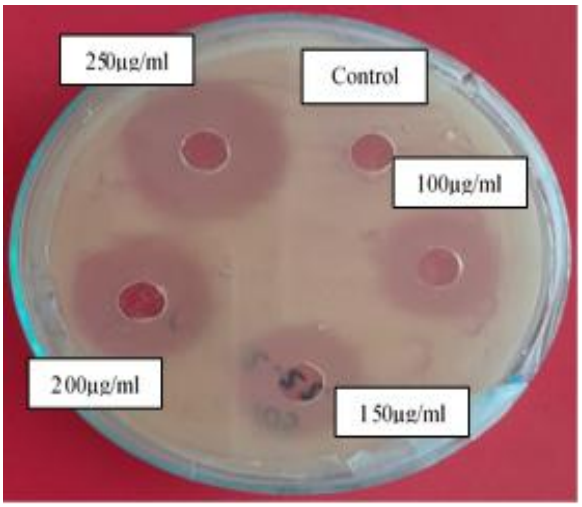

Figure 1: Antibiotic Sensitivity Test of Agrobacterium Strain EHA 105

Figure 2: Carrot discs showing morphologically indistinguishable galls induced by Agrobacterium strain EHA105 (A) and a Control disc (with no Agrobacterium) (B)

Table 1: Callus Induction and Proliferation of Co-cultivated explant

No.	Hormone concentration (mg/L)	Callus Induction	Callus Proliferation
C1	2,4-D 0.5 + Kinetin 1	(+++)	
C2	2,4-D 1 + Kinetin 1	(+)	
C3	2,4-D 0.1 + Kinetin 0.5	(++)	
C4	2,4-D 0.1 + Kinetin 1	(-)	
C5	2,4-D 1 + BAP 0.5		(+++)

Table 2: Effect of *Agrobacterium* Concentration and Co-cultivation Duration on Cotton Transformation Efficiency

Co-cultivation Duration	Agrobacterium Concentration (OD_{600})	Explant Survival Rate (%) (±SE) *	Transformation Efficiency (%) (±SE)
2 Days	0.4	85.0 ± 2.5	1.2 ± 0.5
	0.6	80.5 ± 3.1	3.5 ± 0.8
	0.8	72.0 ± 2.8	8.9 ± 1.1
	1.0	55.5 ± 4.2	2.1 ± 0.7
3 Days	0.4	78.3 ± 3.0	2.5 ± 0.6
	0.6	75.0 ± 2.5	12.3 ± 1.5
	0.8	65.4 ± 3.3	25.6 ± 2.0
	1.0	40.2 ± 4.0	5.4 ± 1.0
4 Days	0.4	70.1 ± 3.5	3.1 ± 0.7
	0.6	60.8 ± 3.8	8.5 ± 1.2
	0.8	45.5 ± 4.1	10.2 ± 1.4
	1.0	25.5 ± 3.9	1.5 ± 0.6

DISCUSSION

In this study, the influence of Agrobacterium density during inoculation and co-cultivation at different periods was examined. Explant survival and bacterial expansion were best controlled when the OD_{600} of the bacterial cells was 0.8. Winter jujube [9], sugarcane [10], and *Acacia crassicarpa* [11] were all successfully treated with the same density of Agrobacterium.

Inoculation length and duration of infection are significant elements in Agrobacterium-mediated transformation. Many plants, like rice [12] and *Withania somnifera* [13], have an inoculation duration of only 15 to 30 min. Nonetheless, the current study discovered that 10 min of inoculation produced the best results, and three days following infection was also optimal for cotton transformation efficiency. A short duration often results in low transformation efficiency because the integration events are incomplete or too few. The optimal callus induction medium for co-cultivated cotton explants was MS medium with 2,4-D (0.5 mg/L) + Kinetin (1.0 mg/L), whereas 2,4-D (1.0 mg/L) + BAP (0.5 mg/L) was the most effective for callus proliferation. Further investigation of antibiotic sensitivity and carrot disc testing of the Agrobacterium strain showed that 250 mg/L Carbenicillin effectively controlled the Agrobacterium strains, and crown gall formation was observed after three weeks.

CONCLUSION

As an agricultural-based economy, Myanmar requires the dissemination of high-quality, genetically pure seeds coupled with modern cultivation techniques. Although Myanmar's natural environment, land, and climate are fundamentally favorable for conducting agricultural activities, making it a suitable country, the threat posed by insect pests has not yet been effectively managed. Therefore, the adoption of advanced *Agrobacterium*-mediated transformation technology holds

significant potential for enhancing the quality and resilience of Myanmar's major crops.

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Author Contributions

KML contributed to the conceptualization and design of the research, conducted the tissue culture experiments, analyzed the data, supervised the project, provided resources, and drafted and revised the manuscript. AAT and HSM conducted the Agrobacterium infection procedures and antibiotic sensitivity bioassays. AS and TTH assisted with tissue culture proliferation and data collection. All authors reviewed and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Use of AI in Drafting of Manuscript

The authors declare that they have not used any generative AI/AI-assisted technologies in the writing of this manuscript.

Conflict of interest

The authors declare no conflict of interest.

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