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Development of Doubled Haploid Lines in Brinjal (*Solanum melongena* L.) via Anther Culture

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Abstract

Brinjal (*Solanum melongena* L.) is an economically important vegetable crop native to Asia, cultivated for over 4,000 years. Despite progress, efficient and reliable protocols for routine application in breeding programs remain elusive for many Solanaceae crops. Anther culture is an effective method for producing doubled haploid (DH) lines in various crops, accelerating breeding programs and genetic research. In this study, anther cultures from three brinjal genotypes were evaluated using standardized media (BR-I initiation and BR-R regeneration media). A total of 31 regenerated plants were evaluated for ploidy levels. The embryo induction rate was calculated to be 1.27%, based on the number of embryos per 100 anthers. The regeneration efficiency for each genotype was as follows: Vishal had 14 plants tested with 6 DH (42.86%); VRHBH had 10 tested with 4 DH (40.0%); and Beauty had 7 tested with 2 DH (28.57%). The use of reported media proved to be more effective for embryogenesis and double DH production compared to modified media.

Keywords: *Solanum melongena*, brinjal, Anther Culture, Doubled Haploid (DH), Embryo Induction, Regeneration Efficiency, Genotypic Response.

1. Introduction

Brinjal (*Solanum melongena* L.), also known as eggplant, is an economically important vegetable crop native to India or Asia, cultivated for over 4,000 years. It is widely cultivated in tropical, subtropical, and warm-temperate regions [1-2]. Brinjal has significant nutritional value, containing amino acids, alkaloids, pigments, flavonoids, and sugars. It also has medicinal properties, used in Ayurveda for treating diabetes, liver complaints, and controlling serum cholesterol [1, 3]. The crop faces various biotic stresses from fungi, bacteria, viruses, mycoplasma, and nematodes, which can reduce its quality and market value [4].

Genetic diversity studies have been conducted on brinjal to understand its crop biology and agricultural aspects [2, 5].

Double-haploid (DH) production in Solanaceae has been extensively studied, with varying success across different species. Tobacco is highly responsive, while tomato remains recalcitrant [6]. Anther culture has shown promise in eggplant and pepper, but isolated microspore culture, a more efficient technique, is still underdeveloped [7]. In eggplant, another culture is currently the most valuable method for DH production, although an isolated microspore culture is recommended when possible to avoid potential regeneration from somatic tissues [8]. Various stress pre-treatments, such as cold treatment, centrifugation, and osmotic shock, have been applied to enhance androgenesis in recalcitrant species [9]. Despite progress, efficient and reliable protocols for routine application in breeding programs remain elusive for many Solanaceae crops. Continued research into embryo development and alternative technologies may help overcome current limitations in DH production for these economically important species [8, 10].

Homozygous lines are crucial in brinjal breeding for developing high-yielding F1 hybrids [8]. Traditionally, these lines are obtained through several generations of self-crossing, which is time-consuming and expensive [10]. Doubled haploid (DH) technology offers a faster alternative, producing homozygous lines in a single generation [8]. Anther culture is currently the most effective method for DH production in eggplant, although isolated microspore culture is recommended when possible to avoid potential regeneration from somatic tissues [8]. However, eggplant remains moderately recalcitrant to DH production, with genotype-dependent success rates [10]. Recent advances in embryo development knowledge are improving protocols for recalcitrant solanaceous crops [10]. Conventional breeding programs focus on yield improvement, stress tolerance, and enhanced nutritional content, while genomic tools are increasingly supporting marker-assisted selection [11]. Anther culture is an effective method for producing doubled haploid (DH) lines in various crops, accelerating breeding programs and genetic research. Studies have demonstrated successful protocols for wheat (Grauda *et al*., [12], rice (Gunarsih *et al*.) [13], oats (Kiviharju *et al*.) [14] and cauliflower (Bhattacharya *et al*.)[15]. Key factors influencing DH production include genotype, culture media composition, and pre-treatment conditions.

2. Methodology

All experimental procedures were carried out at the Research Centre and the Kalash Pvt. Ltd. Laboratory, under controlled environmental conditions designed to optimize doubled haploid (DH) production in *Solanum melongena* L. (brinjal).

Plant Material:

A total of three donor genotypes of *Solanum melongena* L. (brinjal) were cultivated under controlled environmental conditions. Flower buds at the late uninucleate to early binucleate stage were selected for doubled haploid (DH) production. Collected buds were surface sterilized using 5% sodium hypochlorite solution supplemented with 2–4 drops of Tween 20 for 15 minutes, followed by three rinses with sterile distilled water under aseptic conditions.

Culture Media Preparation:

The culture media used in the experiment were prepared with specific formulations tailored for different stages of anther culture and plant regeneration. The embryo induction medium (initiation medium) was composed of MS-B5 powder (4.41 g $\cdot L^{-1}$), supplemented with 2 mg $\cdot L^{-1}$ of kinetin, 2 mg $\cdot L^{-1}$ of 2,4-dichlorophenoxyacetic acid (2,4-D), 120 g $\cdot L^{-1}$ of sucrose, and 9.2 g $\cdot L^{-1}$ of agar. For embryo regeneration, the B&R regeneration medium was prepared using a BR stock solution enriched with 1 mg $\cdot L^{-1}$ of vitamin B_{12}, 1 mg $\cdot L^{-1}$ of kinetin, 30 g $\cdot L^{-1}$ of sucrose, and 9.2 g $\cdot L^{-1}$ of agar. The embryo development medium shared the same composition as the regeneration medium and was employed for further embryo growth. Finally, for plantlet conversion, an embryo maturation medium was used, which contained MS stock solution, 15 g $\cdot L^{-1}$ of

sucrose, and 8 g·L^{-1} of agar to support complete plantlet development [16-21].

Anther Culture:
Surface-sterilized anthers were cultured on embryo induction medium and incubated at 32 °C in the dark for 8 days. Anthers were transferred to B&R medium and incubated at 25 °C in the dark. Subculturing was performed after 12, 21, and 21 days, respectively. Embryo induction continued on B&R medium under 25 °C with a 16/8 h light/dark photoperiod for 90 days. Developed embryos were transferred to a half-strength MS medium and incubated at 25 °C under light for 25 days. The ploidy status of regenerated plantlets was analyzed to identify true doubled haploids. Confirmed DH plantlets underwent primary hardening in growth chambers for 25–30 days, followed by secondary hardening in greenhouse conditions for 4–5 months. Seeds were harvested from mature, selfed DH plants [22-24].

3. Results and Discussion

Regeneration Optimization on Reported Media: Anther cultures from three brinjal genotypes 'RB 9-2-1-1 IR', 'VRHBH RB 9-2-1-1 IR', and 'Beauty RB 9-2-1-1 IR' were assessed using standardized media (BR-I initiation and BR-R regeneration media). A total of 3,000 anthers and 600 donor buds (200 per genotype) were cultured (Table 1). Embryo induction occurred in all three genotypes, with varying efficiency: 'Vishal' exhibited the highest rate of embryo induction, producing 16 embryos (1.6%), while 'VRHBH' yielded 12 embryos (1.2%) and 'Beauty' produced 10 embryos (1.0%). The overall embryo induction rate was calculated to be 1.27%, based on the number of embryos per 100 anthers. The efficiency of subculturing indicated a greater number of embryos formed during the first and second subculture stages, suggesting that successive subcultures contribute positively to the embryogenic response.

Plant regeneration and ploidy results:
A total of 31 regenerated plants were evaluated for ploidy levels. Among them, 61.29% were haploids (N), while 38.71% were confirmed as doubled haploids (DH). No mixoploids were detected. The regeneration efficiency for each genotype was as follows: Vishal had 14 plants tested with 6 DH (42.86%); VRHBH had 10 tested with 4 DH (40.00%); and Beauty had 7 tested with 2 DH (28.57%). These results indicate that the combination of BR-I and BR-R media demonstrates moderate efficiency for DH production through anther culture, with 'Vishal' emerging as the most responsive genotype

Optimization of Modified Media for Efficient Regeneration
To enhance embryogenesis and double haploid (DH) yield, modified media (Mod-B-I to Mod-B-III for initiation and Mod-R-I to Mod-R-III for regeneration) were evaluated across the same genotypes (Table 2). A total of 9,000 anthers and 1,800 buds were cultured (nine treatments, each with 1,000 anthers). Only eight embryos were induced across all combinations of modified media, resulting in an overall embryo induction rate of 0.27%, which is significantly lower than the rates reported for the BR-I/BR-R media. Embryo development was observed exclusively in the following combinations: Mod-B-II with Mod-R-I, producing one embryo (Vishal); Mod-B-III with Mod-R-I, yielding four embryos (Vishal); and Mod-B-III with Mod-R-II, resulting in three embryos (VRHBH). No embryos were recorded for the 'Beauty' genotype under any of the modified media.

Ploidy Testing Results (Modified Media):
Among the three regenerated plants, all were successfully confirmed as doubled haploid (DH) plants, resulting in a 100% DH conversion rate for those that regenerated. However, the low regeneration rate poses limitations for broader applications.

In related studies, numerous researchers have investigated another culture techniques to optimize DH production across various plant genotypes. Gioi and Tuan [25] demonstrated that the N6 medium, when combined with specific plant growth regulators, produced the highest callusing and regeneration rates, particularly in the IR64/IR68530 cross, achieving a regeneration efficiency of 5.73%.

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Table 1: Regeneration Optimization in Different Brinjal Genotypes on Reported Media

Sr. No	Name of Genotype	Lab Code	Reported I-Media	No. of Anthers Initiated	No. of Buds	Reported R-Media	Subculture-I	Subculture-II	Embryo Induction	No. of Shoots Subcultured	No. of Plants Tested for Ploidy	No. of N Plants	No. of DH Plants	Mix Plant	Variety-wise Embryo Induction (%)
1	Vishal RB 9-2-1-1 IR	VISH	BR-I	1000	200	BR-R	650	600	16	14	14	8	6	0	1.6
2	VRHBH RB 9-2-1-1 IR	VHB	BR-I	1000	200	BR-R	550	490	12	10	10	6	4	0	1.2
3	Beauty RB 9-2-1-1 IR	BU	BR-I	1000	200	BR-R	590	525	10	7	7	5	2	0	1.0
	Total			**3000**	**600**				**38**	**31**	**31**	**19**	**12**	**0**	

Note:

Overall Percentage of Embryo Induction = 1.27%

Regenerated Plant to N (haploid) Plant Induction Rate = 61.29%

Regenerated Plant to DH (doubled haploid) Induction Rate = 38.71%

Table 2: Optimization of Different Modified Media for Efficient Regeneration

Sr. No	Name of Genotype	Lab Code	Media Code for Initiation	No. of Anthers Initiated	No. of Buds	Media Code for Subculture	Subculture-I	Embryo Induction	No. of Shoots Subcultured	No. of Plants Tested for Ploidy	No. of N Plants	No. of DH Plants	Mix Plant	Percentage of Embryo Induction (%)
1	Vishal RB 9-2-1-1 IR	VISH	Mod-B-I	1000	200	Mod-R-I	40	0	0	0	0	0	0	0.0
2	VRHBH RB 9-2-1-1 IR	VHB	Mod-B-I	1000	200	Mod-R-II	35	0	0	0	0	0	0	0.0
3	Beauty RB 9-2-1-1 IR	BU	Mod-B-I	1000	200	Mod-R-III	50	0	0	0	0	0	0	0.0
4	Vishal RB 9-2-1-1 IR	VISH	Mod-B-II	1000	200	Mod-R-I	100	1	0	0	0	0	0	0.0
5	VRHBH RB 9-2-1-1 IR	VHB	Mod-B-II	1000	200	Mod-R-II	95	0	0	0	0	0	0	0.0
6	Beauty RB 9-2-1-1 IR	BU	Mod-B-II	1000	200	Mod-R-III	120	0	0	0	0	0	0	0.0
7	Vishal RB 9-2-1-1 IR	VISH	Mod-B-III	1000	200	Mod-R-I	380	4	2	2	2	0	0	0.4
8	VRHBH RB 9-2-1-1 IR	VHB	Mod-B-III	1000	200	Mod-R-II	250	3	1	1	1	0	0	0.3
9	Beauty RB 9-2-1-1 IR	BU	Mod-B-III	1000	200	Mod-R-III	300	1	0	0	0	0	0	0.1
	Total			**9000**	**1800**			**8**	**3**	**3**	**3**	**0**	**0**	

Similarly, Bhattacharya *et al.* [15] reported a callus induction success rate of 1.52% from 20,300 anthers of a rice hybrid, with 0.48% developing into green plants. This highlights the significance of media composition in DH production. Furthermore, Sharma *et al.* [26] optimized conditions for callus induction using N6 medium, attaining a maximum callus induction frequency of 9.39% through the use of 2.5 mg/L of 2,4-D and a 10-day cold pretreatment. Collectively, these studies underscore the crucial role of media formulation and growth regulators in enhancing embryogenic responses and improving DH production efficiency within anther culture systems.

Conclusion

The use of reported C, R and V3 media has proven to be more effective for anther-derived embryogenesis and double haploid (DH) production than modified media. Among the genotypes tested, 'Vishal' consistently displayed a higher responsiveness in both embryo induction and DH conversion. Modified media showed limited effectiveness and are not recommended without further optimization. Therefore, the combination of optimized subculturing timing and the selection of responsive genotypes such as 'Vishal' is critical for enhancing DH production protocols in brinjal. These findings underscore the vital importance of media composition, hormonal balance, and pretreatment conditions in maximizing embryogenic outcomes and DH recovery within plant tissue culture systems.

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