ASSESSMENT OF CRITICAL PARAMETERS ASSOCIATED WITH IN PLANTA TRANSFORMATION OF ELITE SOYBEAN GENOTYPE(S) (*Glycine max* L.)

THESIS

Submitted to
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
in partial fulfillment of the requirements
for the Degree of

MASTER OF SCIENCE
IN
AGRICULTURE
(MOLECULAR BIOLOGY AND BIOTECHNOLOGY)

By

ZANJAL SAGAR LAXMAN

BIOTECHNOLOGY CENTRE
DEPARTMENT OF AGRICULTURAL BOTANY
POST GRADUATE INSTITUTE, AKOLA

DR. PANJABRAO DESHMUKH KRISHI VIDYAPEETH,
KRISHINAGAR PO, AKOLA (MS) 444104

Enrolment Number – QQ-143 **2024**

DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation in the thesis entitled **"ASSESSMENT OF CRITICAL PARAMETERS ASSOCIATED WITH IN PLANTA TRANSFORMATION OF ELITE SOYBEAN GENOTYPE(S) (*Glycine max* L.)"** or part thereof has neither been submitted for any other degree or diploma of any university, nor the data have been derived from any thesis / publication of any university or scientific organization. The source of material used and all assistance received during the course of investigation have been duly acknowledged.

(Zanjal Sagar Laxman)

Place: Akola **Enrolment No. QQ-143**

Date: / / 2024

CERTIFICATE

This is to certify that thesis **"ASSESSMENT OF CRITICAL PARAMETERS ASSOCIATED WITH IN PLANTA TRANSFORMATION OF ELITE SOYBEAN GENOTYPE(S) (*Glycine max* L.)"** submitted in partial fulfillment of the requirement for the degree of **"Master of Science in Agriculture (Molecular Biology and Biotechnology)"** of the Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **Zanjal Sagar Laxman** under my guidance and supervision.

The subject of the thesis has been approved by the Students Advisory Committee.

Place: Akola
Date: / /2023

(Dr. P. V. Jadhav)
Chairman
Advisory Committee

Counter signed

Associate Dean,
Post Graduate Institute, Akola
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola

THESIS APPROVED BY THE STUDENT ADVISORY COMMITTEE
INCLUIDING EXTERNAL MEMBER (AFTER VIVA-VOCE)

1. Chairman	Dr. P. V. Jadhav	______________
2. Member	Dr. M. P. Moharil	______________
3. Member	Dr. D. A. Padole	______________
4. Member	Dr. S. S. Nichal	______________
5. External Member	[ ]	______________

ACKNOWLEDGEMENT

Words, no matter how formal, often fail to convey the true depth of emotions; yet, they remain our only vessel for expressing heartfelt sentiments in this formal acknowledgment. It is with profound gratitude and an overwhelming sense of joy that I extend my sincerest appreciation to my esteemed research guide, Dr. P. V. Jadhav, Assistant Professor, Biotechnology Centre, Department of Agricultural Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. His unwavering dedication, invaluable expertise, and tireless mentorship have been instrumental in guiding me throughout the entirety of this research journey. I am deeply indebted for his consistent encouragement, insightful advice, constructive feedback, and immense patience, all of which have been the cornerstone of my work. His boundless support, timely suggestions, and unwavering belief in my potential have been a source of immense inspiration.

The wisdom and guidance imparted by my mentor will forever illuminate my journey, serving as a guiding light as i navigate the uncharted paths of my future endeavours. I extend my deepest gratitude to the members of my advisory committee: Dr. M. P. Moharil, Associate Professor and In-Charge, Biotechnology Centre, Dr. D. A. Padole, Assistant Professor, Biotechnology Centre, and Dr. S. S. Nichal, Head, RRC Amravati. Their insightful discussions, constructive feedback, and invaluable suggestions have significantly enriched my research work.

I am also profoundly thankful to Dr. S. B. Sakhare, former Associate Professor, Biotechnology Centre, Dr. PDKV, Akola, for his kind cooperation and support in the successful completion of this research. My heartfelt appreciation extends to Dr. A. G. Deshmukh, Assistant Professor, and Dr. D. R. Rathod, Assistant Professor, Department of Agricultural Biotechnology, for their unwavering assistance and wise counsel during this endeavour. I am sincerely grateful to Prof. Dr. T. H. Rathod, Head of the Department of Agricultural Botany, Dr. PDKV, Akola, for the essential facilities provided and for his invaluable suggestions throughout the course of my research. I express my profound gratitude to Dr. Y. B. Tayde, Associate Dean, Post

Graduate Institute, Dr. PDKV, Akola, for facilitating the necessary resources and support for my degree program and the completion of this research work.

I extend my heartfelt appreciation to Dr. Umesh Shinde (JRF), Miss Ruchika Bhagat (JRF), and Dr. Arpita Mahobia for their invaluable cooperation, timely assistance, and unwavering moral support throughout this investigation. My sincere thanks go to my esteemed seniors: Miss Gopika K. Mote (Ph.D. Scholar), Mr. Balaji Jadhav (Ph.D. Scholar), Mr. Shreshtha Saxena (Ph.D. Scholar), Mr. Shubham Patil (Ph.D. Scholar), and Mr. Sachin Abhang (Ph.D. Scholar), as well as my M.Sc. seniors: Rahul Bawankar, Snehal Yermalwar, Akansha Jadhav, Vaibhav Solpure, Amar Gajbhiye, and Rushikesh Giri. Their insightful suggestions, generous guidance, stimulating discussions, and constructive ideas have been instrumental in shaping my research journey. I also acknowledge with gratitude the non-teaching staff of the Biotechnology Centre, Shri R. R. Jirapure and Bhagat Dada, for their indispensable support during my studies.A special mention of gratitude is reserved for my dear batchmates Bhagyashree, Vaishnavi B., Sourabh, Lesha, Samiksha, Vaishnavi Z., Shrikant and my junior companions Sarvesh, Akshay, Tanavi, and Vishal, whose encouragement and assistance were crucial throughout my academic pursuits.

I am forever indebted to my parents, Laxman Z. Zanjal and Nanda L. Zanjal, and my brother, Shreyash L. Zanjal, for their boundless love, unwavering faith, and immeasurable support. My deepest thanks extend to all my family members, whose encouragement has been a constant source of strength. I am grateful to all the authors whose literature has been cited in this thesis, and to everyone who, directly or indirectly, contributed to the accomplishment of this work. Above all, I owe the greatest debt to the divine Almighty, Lord Ganesh, whose blessings and will have guided me to this moment of success. I conclude with heartfelt thanks to one and all.

Place : Akola **(Zanjal Sagar Laxman)**
Date : (Enrol. No. : QQ-143)

Table of Contents

Sr. No.	Particulars	Page
A	Declaration of Student	i
B	Certificate	ii
C	Acknowledgement	iii-iv
D	List of Tables	vi
E	List of Figures	vii
F	List of Plates	viii
G	List of Abbreviations	ix-xi
H	Glossary	xii-xiii
I	Thesis Abstract	xiv-xv
I	Introduction	1-8
II	Review of Literature	9-22
III	Material and Methods	23-37
IV	Results and Discussion	38-63
V	Summary and Conclusions	64-65
VI	Implications	66
VII	Literature Cited	67-73
	Vita	74
	Appendix	75-80

(A)List of Tables

Sr. No.	Title	Page
1.1	State-wise area, yield, and production of soybean in India (Kharif 2023-24)	2
3.1	Soybean genotypes used in the present investigation	23
3.2	Treatments of *Agro*-injection and Sonication using half and full seed in Soybean	28
3.3	Composition of different solutions required for plasmid isolation	29
3.4	Composition of CTAB buffer	33
3.5	Primer sequence used for screening of putative transformants	33
3.6	Temperature profile for amplification of gRNA specific primers	34
3.7	PCR Temperature Profile for Amplification of gRNA-Specific and Cas9-Specific Primers	34
3.8	PCR components and their volumes in a reaction mixture	34
3.9	Recorded observations	36
4.1	Effect of full-seed sonication treatments on seed germination in experimental soybean genotypes	41
4.2	Effect of Half-Seed sonication treatments on seed germination in different soybean genotypes	43
4.3	Effect of Argo-injection at embryo on seed germination in experimental soybean genotypes	45
4.4	Percentage of Pod Formation from Different Agro-Injection Treatments at Floret Stage	49
4.5	PCR screening of plants derived from full-seed sonication Treatments	58
4.6	PCR positive plants derived from half-seed sonication treatment	59
4.7	PCR positive for every treatment of Agro-injection at Embryo	60
4.8	PCR positives plants derived from Agro-Injection treatments at Floret	61
4.9	Response of experimental soybean genotypes to In-Planta transformation	62

(B) List of Figures

Sr. No.	Title	Page
3.1	Partial map of T-DNA of vector p_DIRECT_22A having gRNA to target GmBADH2 gene	25
3.2	Partial map of T-DNA of vector p_DIRECT_22A having gRNA to target GmKTI gene.	25
4.1	Effect of full-seed sonication treatment on seed germination in soybean genotypes	42
4.2	Effect of Half-seed sonication treatments on seed germination in different soybean genotypes	44
4.3	Effect of Agro-injection embryo treatments on seed germination in experimental soybean genotypes	47
4.4	Pod bearing percentage in response to Agro-injection at floret across different treatment	48
4.5	Graphical representation of the effect of sonication on explants	52
4.6	Response of experimental soybean genotypes to *In-Planta* transformation	63

(C) List of Plates

Sr. No.	Caption	Page
4.1	Confirmation of recombinant strain by colony PCR using sgRNA-specific primers targeting the BADH2/KTI genes	40
4.2	Effect of sonication at different time interval on cotyledon of AMS-100-39	46
4.3	Target floral Sites for *Agro*-injection in Soybean	51
4.4	Screening for putative transformants using sgRNA-specific primer	53
4.5	Screening for putative transformants using Cas9-specific primer	56

(D) LIST OF ABBREVIATIONS

/ : Per

% : Per cent

± : Plus or minus

^{0}C : Degree Celsius

pH : Hydrogen ion concentration

LAF : Laminar air flow

GM : Genetically modified

GE : Genome editing

SAAT : Sonication-Assisted Agrobacterium-Mediated Transformation

GiFT : Genotype-independent fast transformation

DNA : Deoxyribose nucleic acid

2AP : 2-acetyl-1-pyrroline

BADH : Betaine aldehyde dehydrogenase

KTI : Kunitz Trypsin Inhibitor

TIs : Trypsin inhibitors

kDa : kilodalton

CRISPR : Clustered regularly interspaced short palindromic repeats

Cas9 : CRISPR associated protein 9

CH : Casein hydrolysate

µM : Micromolar

N : Normality

PCR : Polymerase chain reaction

FCRD : Factorial completely randomized design

IAA : Indole-3-acetic acid

et al.	:	Et. Alibis (and associates)
Fig.	:	Figure
GA3	:	Gibberellic acid
@	:	At the rate
v/v	:	Volume by volume basis
w/v	:	Weight by volume basis
ml	:	Milliliter
g	:	gram
mg	:	Milli liter(s)
mg/L	:	Milligram per litter
SNP	:	Single nucleotide polymorphisms
SNP	:	Sodium nitroprusside
HCl	:	Hydrochloric acid
NaOH	:	Sodium hydroxide
HEPA	:	High Efficiency Particulate Air
RAPD	:	Random Amplified Polymorphic DNA
bp	:	base pairs
i.e.	:	That is
mRNA	:	messenger Ribonucleic Acid.
TIA	:	trypsin inhibitor activity
BBi	:	Bowman–Birk protease inhibitor
Ha	:	Hectare
S.E.	:	Standard Error
CD	:	Critical Difference
SV	:	Swarna Vasundhara

LAF	:	Laminar Air Flow
OD	:	Optical density
DMSO	:	Dimethyl sulfoxide
MCT	:	Micro Centrifuge Tube
CTAB	:	cetyltrimethylammonium bromide
pmol	:	picomoles per liter
NFW	:	Nuclease free water
Sec	:	Second

(E) Glossary

Agro-injection	**:**	A genetic transformation technique where *Agrobacterium tumefaciens* is directly injected into plant tissues to deliver DNA.
BADH2	**:**	Betaine aldehyde dehydrogenase 2, a gene involved in the biosynthesis of 2-acetyl-1-pyrroline (2AP), a compound associated with aroma in plants.
Cas9	**:**	CRISPR-associated protein 9, an enzyme used in genome editing to introduce targeted cuts in DNA.
CRISPR	**:**	Clustered Regularly Interspaced Short Palindromic Repeats, a genome-editing technology used for precise genetic modifications.
CTAB	**:**	Cetyltrimethylammonium bromide, a reagent used in DNA extraction to lyse cells and precipitate nucleic acids.
DNA (Deoxyribonucleic Acid	**:**	The molecule carrying genetic instructions for the development, functioning, growth, and reproduction of all known organisms.
Embryo	**:**	The early developmental stage of a plant, where cells have the potential to develop into any tissue type.
gRNA:	**:**	Guide RNA, a component of the CRISPR system that directs the Cas9 enzyme to specific locations in the genome for editing.
In-planta Transformation	**:**	A method of genetic modification where DNA is delivered directly into plant tissues, bypassing traditional tissue culture steps.
KTI (Kunitz Trypsin Inhibitor)	**:**	A protein that inhibits trypsin activity, reducing protein digestibility in soybeans.
PCR (Polymerase Chain Reaction)	**:**	A molecular biology technique used to amplify DNA sequences, making them easier to study.
Pod Bearing Percentage	**:**	A parameter indicating the number of pods formed per number of flowers, used to assess the success of transformation methods.
SAAT (Sonication-Assisted Agrobacterium-Mediated	**:**	A technique that uses ultrasound to create microwounds in plant tissues, enhancing the efficiency of *Agrobacterium*-mediated transformation.

Transformation)

Sonication	**:**	The use of sound waves to agitate particles in a sample, often used to facilitate DNA uptake during transformation.
T-DNA	**:**	Transfer DNA, a segment of DNA transferred from *Agrobacterium* to a plant during genetic transformation
Transformation Efficiency	**:**	The percentage of plants successfully incorporating foreign DNA out of the total number of treated plants.

F) THESIS ABSTRACT

a) Title of the thesis : **ASSESSMENT OF CRITICAL PARAMETERS ASSOCIATED WITH IN PLANTA TRANSFORMATION OF ELITE SOYBEAN GENOTYPE(S) (*Glycine max* L.)**

b) Full name of student : **Zanjal Sagar Laxman**

c) Name and address of Major Advisor : **Dr. P. V. Jadhav**

Assistant Professor, Biotechnology Centre, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola

(M.S.) – 444 104.

d) Degree to be awarded : M.Sc. in Agriculture

(Agricultural Biotechnology)

e) Year of award of degree : 2024

f) Major subject : Molecular biology and biotechnology

g) Total number of pages in the thesis : 66

h) Number of words in the abstract : 240

i) Signature of student :

j) Signature, Name and address of forwarding authority :

Head
Department of Agricultural Botany
Post Graduate Institute,
Dr. PDKV, Akola

ABSTRACT

The present study, titled "Assessment of Critical Parameters Associated with *In-Planta* Transformation of Elite Soybean Genotypes (*Glycine max* L.)," aimed to evaluate the efficacy of two *in-planta* transformation methods Sonication-Assisted *Agrobacterium*-Mediated Transformation and *Agro*-Injection and the critical parameters influencing their success. Conducted at the Biotechnology Centre, Dr. PDKV, Akola, during 2022–23, the study sought to address the challenges of soybean's recalcitrant nature and low transformation efficiency. Transformation experiments targeted two organs, the embryo and ovary, using three soybean genotypes: one vegetable-type, Swarna Vasundhara (SV), and two grain-type varieties, Swarna Soya (AMS MB 5-18) and PDKV Yellow Gold (AMS 1001). *Agrobacterium* tumefaciens strain EHA 105, harboring the plasmid vector pDIRECT_22A with sgRNAs targeting BADH2 and KTI genes, was used. Colony PCR with sgRNA-specific primers confirmed the integration of the constructs. Critical parameters, including germination percentage, pod-bearing rate, and CRISPR/Cas9 delivery efficiency, were assessed. Scanning Electron Microscopy (SEM) analysis of sonicated embryos revealed an increase in the number and size of micro-wounds with prolonged sonication time. Molecular screening using PCR demonstrated that transformation efficiency varied significantly across genotypes and techniques. The highest efficiency (3.3%) was achieved in PDKV Yellow Gold using the full-seed sonication method, highlighting the genotype's receptivity to this approach. *Agro*-injection methods showed lower and inconsistent efficiencies, with a maximum of 2.6% in Swarna Vasundhara targeting the ovary at the floret stage. These results emphasize the need for genotype-specific optimization of transformation protocols to enhance transformation outcomes in soybean.

CHAPTER I

INTRODUCTION

1.1 Background Information

Soybean (*Glycine max* L.Merr) belonging to the Fabaceae family is long acknowledged as a valuable crop contributing to over 52 million metric tons grown production worldwide. This crop plays a crucial role in the Agro-economy, serving as a staple in human nutrition, animal feed, and various industrial applications, also accounts for more than 60% of all oilseed production worldwide (USDA FAS Report 2024). It contains about 40% protein and 20% vegetable oil which exceeds more than that of any other legume crops and because of its amino acid composition, soy protein is also called a complete protein.

Despite its rich composition, soybean consumption faces numerous challenges, including anti-nutritional factors and off-flavor. To address these challenges, there has been a concerted effort to enhance soybean's genetic makeup through biotechnological interventions.

1.2 Area and cultivation of soybean

World soybean production in 2023-24 was estimated at 394.71 million tonnes from a total area of 136.82 million hectares. Brazil ranked first in soybean production with 153 million tonnes, followed by the United States (113.27 million tonnes), Argentina (48.10 million tonnes), China (20.84 million tonnes), and India (11.88 million tonnes), accounting for 39%, 29%, 12%, 5%, and 3% of the world's total production, respectively. In terms of area, India ranked fourth with 12.12 million hectares (29.94 million acres), representing 8.86% of the global soybean area, and fifth in production with 11.23 million tonnes (U.S. Department of agriculture).

The major soybean-growing states in India include Madhya Pradesh, Maharashtra, Rajasthan, Karnataka, and Telangana. According to the first advance estimates for 2023-24 from the Government of India, soybean production is expected to be 115.28 lakh tonnes, down from 149.85 lakh tonnes in 2022-23. Among the states, Madhya Pradesh leads in production with 45.97 lakh tonnes, followed by Maharashtra (45.74 lakh tonnes), Rajasthan (10.69 lakh tonnes), Karnataka (4.73 lakh tonnes), Gujarat (4.23 lakh tonnes), and Telangana (2.90 lakh tonnes).

Table 1.1. State-wise area, yield, and production of soybean in India (Kharif 2023-24)

Sr. No.	Name of State	Area (lakh ha)	Production (lakh MT)
1	Madhya Pradesh	53.35	45.97
2	Maharashtra	50.72	45.74
3	Rajasthan	11.44	10.69
4	Karnataka	4.11	4.73
5	Gujrat	2.66	4.23
6	Telangana	1.89	2.90
7	Others	1.44	1.02
	Grand Total	**125.61**	**115.28**

(www.agricoop.gov.in)

1.3 Genetic transformation in Soybean

Genetic transformation of soybean has progressed significantly since its beginning, playing a pivotal role in enhancing traits such as herbicide resistance, pest tolerance, and improved nutritional quality. The journey began in the late 1980s with the first successful *Agrobacterium*-mediated transformation by Hinchee et al. (1988), followed by early biolistic approaches (Christou et al., 1988; McCabe et al., 1988). These early methods, despite their pioneering nature, faced challenges of low transformation efficiency and high genotype dependency. Throughout the 1990s and early 2000s, various improvements were made, including the cotyledonary node method, which emerged as a preferred technique due to its simplicity and higher efficiency (Olhoft et al., 2006; Paz et al., 2004). The introduction of transgenic herbicide-resistant soybeans in the 1990s marked a major commercial success, making soybean one of the leading GM crops globally (Pagano and Miransari, 2016). Over the years, traits like insect resistance and enhanced nutritional profiles were introduced, with notable examples being glyphosate-resistant *Glycine max* and the high-oleic acid soybeans developed for better oil quality (Xu et al., 2020; Cai et al., 2023).

In recent years, genome editing (GE) has revolutionized soybean breeding, enabling precise modifications of native genes, moving beyond traditional transgenic approaches (Sim et al., 2023). While *Agrobacterium* remains the preferred method for gene delivery due to its efficiency and low-copy event generation, alternative systems such as *Ochrobactrum*-mediated transformation have shown potential, although they are less commonly adopted (Broothaerts et al., 2005; Cho et al., 2022). Despite advancements, soybean transformation still encounters hurdles like genotype dependency and the need for labour-intensive tissue culture. The emergence of *in-planta* transformation methods offers a promising alternative by bypassing tissue culture and enabling direct gene delivery into seedlings or intact plants (Bent, 2000; Mangena, 2019). Although challenges like low efficiency and chimerism remain, *in planta* approaches provide a fast, genotype-independent option. Efforts to integrate the strengths of both *in vitro* and *in planta* methods have led to the development of new techniques, such as the GiFT system, aiming to enhance transformation efficiency and broaden the applicability across diverse soybean germplasm (Kesiraju et al., 2021; Zhang et al., 2023).

The *in-planta* method refers to the direct transformation of plants without involving tissue culture steps. It is more efficient, less cumbersome, and less time-consuming than the tissue culture method, making it useful for plants lacking tissue culture and regeneration systems. The last decade has seen significant development in *in-planta* transformation methods. The first successful *in-planta* transformation was reported in *Arabidopsis thaliana*, with improved results using the floral dip method, where plants were inoculated at the flowering stage (Bechtold and Bouchez, 1993). Other crops successfully transformed using *in-planta* methods include *Brassica rapa* (Tague, 2006), *Brassica napus* (Wang et al., 2003), *Brassica campestris* (Chen et al., 2019), *A. thaliana* (Das and Joshi, 2011; Nakashima et al., 1998; Shinwari et al., 2000), *Medicago truncatula* (Trieu et al., 2000), *Raphanus sativus* (Curtis, 2001), *Solanum lycopersicum* (Shah et al., 2015), *Glycine max* (Li et al., 2010; Liu et al., 2009), *Melilotus alba* (Hirsch et al., 2010)and *Oryza sativa* (Ratanasut et al., 2017; Supartana et al., 2005), with *A. thaliana* also reported by Harrison et al. (2006). Various types of explants, such as mature seeds, embryos, inflorescences, embryogenic apical meristems, spikelets, and roots, have been used for transforming these plants.

In our study, we utilized two methods for *in-planta* transformation. The first method is Sonication-Assisted Mediated Transformation (SAAT), which enhances the effectiveness of *Agrobacterium*-mediated transformation using ultrasound. The ultrasound generates microwounds both on the surface and deep within the target tissue, facilitating easier access for *Agrobacterium* to the meristematic cell region. SAAT was initially used for delivering naked DNA into tobacco protoplasts (Joeshro and Brunstedt, 1990) and seedlings (Zhang et al., 1991). In soybeans,(Santarém et al.1998) carried out transformation using sonication method in immature cotyledons and assessed various parameter regarding to it. The efficiency of SAAT can potentially be increased by optimizing co-cultivation conditions, *Agrobacterium* concentration, and sonication parameters. Overall, this technique has shown promise for plant genetic transformation and may contribute significantly to agricultural advancements.

The second method we employed is *Agro*-injection, a commonly used approach for *Agrobacterium*-mediated genetic modification of soybeans. In this technique, *Agrobacterium* carrying the desired gene is directly injected into soybean hypocotyls or cotyledons. The success of *Agro*-injection depends on several factors, such as the strain of *Agrobacterium* used, the extent and duration of the infection, the age and physiological condition of the explants, and various other parameters. Studies have reported successful *Agro*-injection based transformation in soybeans, with transgenic traits passed on through progeny seeds. Compared to traditional tissue culture-based techniques, *Agro*-injection offers advantages like a shorter turnaround time, reduced labour costs, and a lower risk of somaclonal variation. However, achieving consistent and reliable results requires careful optimization of various parameters. Numerous research articles have detailed how these parameters can be fine-tuned, establishing *Agro*-injection as a valuable tool for the genetic improvement of soybeans.

1.4 Targeted Traits

Aroma is one of the desirable quality traits in soybean. Similar to rice-eating communities in Asia and Europe, there is a preference for soybean cultivars with aroma. Research suggests that the presence of specific chemical compounds, such as 2-acetyl-1-pyrroline (2AP), contributes to the fragrance in rice(Bradbury et al., 2005; Fitzgerald et al., 2009). It was also found that in other plants where 2AP is synthesized, the gene responsible for 2AP biosynthesis and mechanism of 2AP was found same having deletions in BADH2 at different locations in the gene. In the aromatic vegetable soybean, the quantitative trait loci (QTL) confirmed the position of fragrance and GmBADH2 for 2AP biosynthesis. It was found that there are three single nucleotide polymorphism (SNP) changes in exon 6 and 10. It was also found that the nucleotide substitution of G to A in exon 10 leads to a change of amino acid from glycine (GGC; G) to aspartic acid (GAC; D) that results in becoming GmBADH2 non-functional (Juwattanasomran et al., 2010).

Trypsin inhibitors (TIs) are major anti-nutritional factors in soybean, limiting its nutritional value due to their ability to inhibit proteolytic enzymes, leading to reduced protein digestion and absorption. The two primary serine protease inhibitors found in soybean seeds are the Kunitz Trypsin Inhibitor (KTI) and Bowman-Birk Inhibitor (BBI). Kunitz first isolated and crystallized KTI from soybean seeds in 1947, identifying it as a non-glycosylated protein weighing 21.5 kDa, with 181 amino acid residues. KTI's strong inhibition of trypsin is attributed to two disulfide bridges (Cys39-Cys86 and Cys138-Cys145), which contribute to its resistance against thermal and chemical denaturation (Roychaudhuri et al., 2004). This inhibitory action disrupts protein digestion in animals, often leading to growth inhibition and pancreatic hypertrophy (Choi et al., 2022). BBI, identified as a smaller 8 kDa protein consisting of 71 amino acids, acts as a dual inhibitor of trypsin and chymotrypsin, and has shown potential in suppressing carcinogenesis in animal studies (Mittal et al., 2021).

The KTI gene family in soybean consists of 38 members spread across 9 chromosomes, with the largest clusters on chromosome 8, containing 13 KTI genes. Notably, specific seed-expressed KTI genes such as Gm01g095000 (KTI1) and Gm08g341000 (KTI3) are linked to anti-nutritional activity (Wang et al., 2022). The presence of KTI poses a challenge in utilizing the full nutritional potential of soybean, necessitating processing methods like heat treatment to inactivate the inhibitors. Recent advancements in soybean breeding and genome editing have focused on developing KTI-free or low-KTI cultivars to enhance the digestibility and nutritional quality of soybean-based products. This progress highlights the importance of continued efforts to reduce anti-nutritional factors in soybean and maximize its benefits as a protein-rich crop.

1.5. Objective

1. To decide the efficiency of sonication and half seed *Agro*-injection based *in-planta* delivery of CRISPR/Cas9 construct for BADH2/KTI gene in elite soybean genotypes
2. Molecular screening of putative transformants using PCR

1.6 Scope and limitations of study

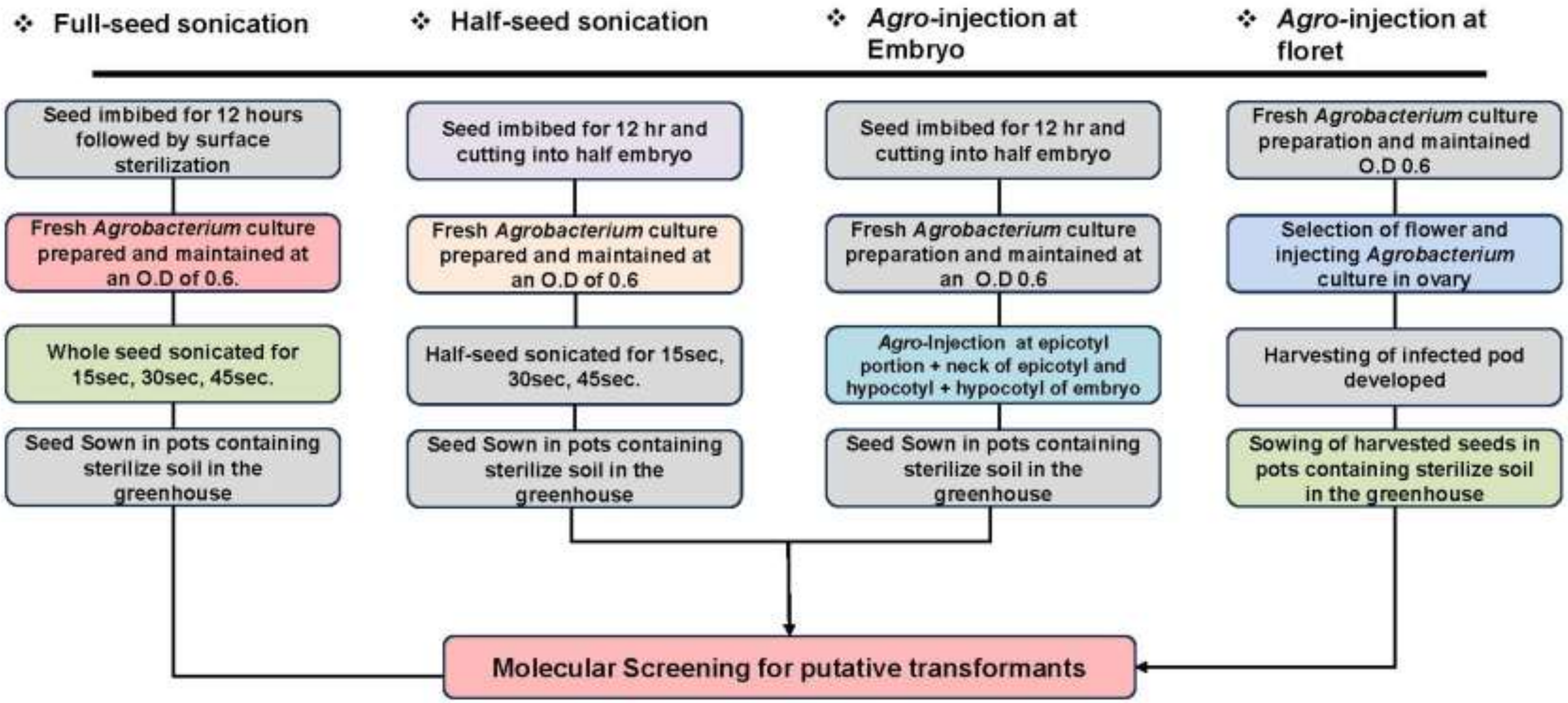
Biotechnology encompasses a range of tools that enable scientists to genetically characterize or enhance living organisms. Emerging technologies such as molecular characterization and genetic transformation are extensively utilized to improve plants. These biotechnological tools complement classical breeding methods by enabling new discoveries and to enhanced nutritional value and increased yields through improved resistance to diseases, herbicides, and abiotic stresses. In soybean breeding programs, both public and private, biotechnology has played and will continue to play a crucial role (Sudaric et al., 2009).

In-planta transformation is an efficient, rapid, and tissue culture-independent system for improving crop plants. This method is particularly useful for plants that lack established tissue culture and regeneration systems. Consequently, direct *In-planta* methods are commonly employed to introduce important genes into such plant species. The two most common *Agrobacterium*-mediated *In-planta* methods, floral dip and vacuum infiltration, have been successfully used by many researchers in both dicot and monocot plants. The primary advantages of *In-planta* transformation include the ability to produce a large number of transgenic plants and the accumulation of high concentrations of total soluble protein in a short period. However, challenges such as the production of chimeric plants and the lack of stable transgene expression at the T1 stage or beyond remain, necessitating further improvements. This investigation focuses on *In-planta* transformation protocols and various parameters that involved in it.

1.7 Hypothesis

In-planta transformation in soybean provides an efficient, cost-effective, and tissue culture-free approach to gene editing, particularly suited for delivering CRISPR/Cas9 constructs. By systematically evaluating key parameters of transformation methods such as sonication and *Agro*-injection, that it is possible to identify an optimal delivery system that maximizes transformation efficiency. Specifically, factors including sonication duration, bacterial strain concentration, injection pressure, and tissue type are expected to significantly influence the effectiveness of construct delivery and overall transformation success. Understanding and refining these parameters will likely result in a more consistent and reliable CRISPR/Cas9 application in soybean.

Furthermore, this approach may lead to the successful development of transformed soybean plants with desirable traits, such as null KTI and enhanced aroma.



In planta transformation

CHAPTER II

REVIEW OF LITERATURE

2.1 Background

2.1.1 Soybean

Soybean (*Glycine max*), annual legume of the pea family (Fabaceae) and its edible seed and are one of the most important sources of protein and oil for human and animal consumption worldwide. Modern cultivars descended from wild soybean (*Glycine soja* Sieb. & Zucc.) in China approximately 5000 years ago (Wu *et al.*, 2024).

2.1.2 Nutrient content

Soybeans have a high protein quality, making soybeans and their food products excellent plant-based protein sources. The soybeans contain ~35–40% protein, ~20% lipids, ~9% dietary fiber, and ~8.5% moisture based on the dry weight of mature raw seeds (Qin *et al.*,2022)

Soybeans are a nutrient-dense food, providing a rich source of various vitamins and minerals. They contain significant amounts of molybdenum, an essential trace element (Hattori et al., 2004), and vitamin K1 (phylloquinone), which is crucial for blood clotting (Shea *et al.*,2008).

Additionally, soybeans are abundant in folate (vitamin B9), important for numerous bodily functions and particularly vital during pregnancy. They also supply copper, a mineral often deficient in Western diets, impacting heart health, and manganese, although its absorption is hindered by phytic acid. Furthermore, soybeans are a good source of phosphorus, essential for numerous physiological processes, and thiamine (vitamin B1), which is critical for many bodily functions.

Soybeans contain significant amounts of phytic acid, dietary minerals and B vitamins. Soy vegetable oil, used in food and industrial applications, is another product of processing the soybean crop. Soybean is the most important protein source for feed farm animals (that in turn yields animal protein for human consumption).

2.1.3 Classification of soybean

The genus Glycine is one of the most important genera in legume plants. It is divided into two subgenera, Glycine Willd. (perennial) and Soja. The subgenus Soja includes two species: an economically very important crop, the soybean [(*Glycine max* (L.) Merr.)], and its wild annual progenitor, *Glycine soja*. The subgenus Glycine contains 26 wild perennial species. Wild perennial Glycine species have great potential for soybean improvement. These species are extremely diverse morphologically, cytologically, and genomically, grow in very diverse climates, and have a wide geographical distribution. They are a rich source of agronomically useful genes.

The taxonomy of cultivated soybean is as follows:

Kingdom : Plantae – Plants

Subkingdom : Tracheobionta

Super division : Spermatophyta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Rosidae

Order : Fabales

Family : Fabaceae

Genus : *Glycine* Willd.

Species : *Glycine max* (L.) Merr.

2.2 Genome editing progress in soybean

2.2.1 *Agrobacterium* mediated transformation in soybean

Hinchee et al. (1988) performed initial experiments and recovered transformed soybean plants using *Agrobacterium* successfully. Regeneration was achieved through shoot organogenesis on cotyledons of susceptible soybean genotypes. Cotyledon explants were infected with

Agrobacterium tumefaciens pTiT37-SE carrying either pMON9749 (kanamycin resistance and GUS activity) or pTiT37-SE∷pMON894 (kanamycin resistance and glyphosate tolerance) and cultured on shoot induction medium with kanamycin. Analysis showed that about 6% of the shoots were transgenic, confirmed by GUS expression or glyphosate tolerance. Two plants produced offspring with Mendelian single-insert inheritance, demonstrating that *Agrobacterium*-mediated gene transfer can generate transgenic soybean.

Chee et al.(1989) and their team developed an avirulent strain of *Agrobacterium* tumefaciens containing the binary vector pGA482 infected the plumule, cotyledon node and adjacent cotyledon tissues of germinating seeds. Neomycin phosphotransferase (NPT) II enzyme activity was found in the tissues of 16 (R0) soybean plants, suggesting that the T-DNA region of pGA482, containing the plant-expressible Nos-NPT II gene, is expressed at least in somatic tissues was transported. Development of R1 plants from putatively transformed R0 soy plants allowed detection of the transferred Nos-NPT II gene and 0.7% of the surviving inoculated seeds yielded transformed tissues in the R0 plant and about 1/10 of these plants yielded transformed R1 plants a plant with desired traits.

Paz et al. (2004) introduced an improved *Agrobacterium tumefaciens*-mediated transformation method using the "half-seed" explant from mature soybean seeds after overnight imbibition. This approach showed a1.5-fold higher transformation efficiency (3.8%) compared to the traditional cotyledonary node method, without the need for complex wounding steps. Stable transgenic events were confirmed in the T_1 generation using phenotypic assays (Liberty® herbicide) and Southern blot analysis. The simplicity and increased efficiency of the half-seed method offer a valuable alternative for efficient and reliable soybean transformation.

Arun et al. (2015) developed an improved *Agrobacterium*-mediated transformation protocol for soybean cultivar PK 416. They evaluated various parameters, including different *Agrobacterium tumefaciens* strains (LBA4404, EHA101, EHA105 with pCAMBIA1304 plasmid), sonication duration, vacuum infiltration pressure, and vacuum duration using cotyledonary node explants from 7-day-old seedlings. Using a direct organogenesis system, they achieved the highest transformation efficiency of 18.6% by sonicating explants for 20 seconds and vacuum infiltrating for 2 minutes at 250 mmHg in *Agrobacterium* EHA105 suspension. Transgene expression was confirmed by GUS and GFP assays, and integration was verified by PCR and Southern blot. This protocol was also effective for four other cultivars (JS 90-41, Hara Soy, Co 1, Co 2), with efficiencies from 13.3% to 16.6%, making it useful for transforming various soybean cultivars with desirable traits.

Yang et al. (2016) developed a high-efficiency *Agrobacterium tumefaciens*-mediated transformation system for soybeans, using cotyledonary explants and glufosinate as a selective agent. They optimized variables across seven cultivars, identifying YC-1 and YC-2 as the most promising with the highest transient GUS expression and shoot induction frequencies. The optimal *Agrobacterium* cell density was OD 0.8–1.0, with beneficial infection conditions at 23°C and dim light. The infection time did not affect GUS expression. Testing three antioxidant solutions to reduce explant browning, they found 20 μM lipoic acid to be most effective. Using the optimized system, they successfully transferred the bar gene into soybean, achieving a 14.71% transformation efficiency, a more than 50-fold increase from the unoptimized method. Southern blot and T1 progeny analysis confirmed the stable integration and inheritance of the transgene.

Zhang et al. (2016) analyzed soybean gene expression to understand the plant's response to early *Agrobacterium* infection, finding that it triggered both PAMPs-triggered and effector-triggered immunity, upregulated MAPK pathway transcription factors, and increased flavonoid and isoflavonoid biosynthesis, leading to a strong defense response and lower transformation efficiency. To counter this, they used α-aminooxyacetic acid (AOA) and sonication with *Agrobacterium* infection. This approach decreased the expression of key genes involved in isoflavone biosynthesis, plant-pathogen interactions, and cell membrane structure, reducing defense responses and increasing susceptibility to

Agrobacterium. This method improved transformation efficiency, suggesting further measures could enhance soybean transformation.

Li et al. (2017) aimed to enhance *Agrobacterium-mediated* transformation efficiency in soybeans by optimizing infection and regeneration processes. They investigated factors affecting *Agrobacterium* infection efficiency, such as bacterial concentration, explant type, suspension medium, and co-cultivation time, achieving over 96% infection by using *Agrobacterium* at OD600 = 0.6 with a medium containing 154.2 mg/L dithiothreitol to infect half-seed cotyledonary explants co-cultured for 5 days. Varieties Jack Purple and Tianlong 1 showed higher infection efficiencies than six other varieties. They also optimized shoot elongation rates by testing different concentrations of gibberellic acid (GA3) and indole-3-acetic acid (IAA), finding the best results with 1.0 mg/L GA3 and 0.1 mg/L IAA, which increased shoot elongation rates by 18 and 11%, and transformation efficiency to 7% and 10% for Jack Purple and Tianlong 1, respectively. Positive transgenic plants were verified using GUS staining, PCR, herbicide testing, and quantification PCR. This improved protocol offers a valuable reference for enhancing transformation efficiency in other plant species.

Chen et al*.* (2018) investigated the impact of l-glutamine and l-asparagine on improving *Agrobacterium-mediated* transformation efficiency in soybeans. They found that adding these amino acids, individually or combined, to the culture medium significantly increased shoot induction, elongation rates, and overall transformation efficiency. The combination of 50 mg/L l-glutamine and 50 mg/L l-asparagine was most effective, enhancing transformation frequency by reducing the expression of GmPR genes (GmPR1, GmPR4, GmPR5, and GmPR10) and suppressing the plant defense response. This method successfully transmitted the transgene to the T1 generation, offering a valuable approach for soybean genetic engineering.

Karthik et al. (2020) explored the use of Sodium nitroprusside (SNP) to enhance genetic transformation efficiency in soybeans. By adding 30 μM SNP to co-cultivation (with 4.44 μM N6-benzyl adenine), shoot induction (with 4.44 μM BA), and rooting medium (with 4.93 μM indole 3-butyric acid), they achieved a transformation efficiency of 34.6%, compared to 23% without SNP. Transformed plants were confirmed using GUS assays, PCR, and Southern hybridization. Additionally, this method shortened the plantlet development period to 75 days from half-seeds. The study demonstrated that SNP significantly improves regeneration and transformation efficiency in soybeans.

Pareddy et al. (2020) developed an organogenesis-based transformation method for soybeans, achieving an average transformation frequency of 18.7%. This method uses *Agrobacterium*-mediated transformation on split-seed explants with an attached partial embryonic axis from imbibed seeds. The *Agrobacterium* tumefaciens strain EHA105 was more effective than EHA101, yielding higher transformation frequencies and more low copy events. The system employed phosphinothricin acetyl transferase for glufosinate tolerance, with 48% of the T1 progeny showing heritability based on molecular analysis and Liberty® herbicide screening. This method was effective across various genotypes, including some elite lines, and is suitable for both research and commercial transgenic soybean production.

Wang et al. (2022) present a highly efficient soybean transformation method using mature seed halves as explants, removing cotyledonary leaves. Optimal media composition and desiccation co-culture treatment on filter paper led to a significant increase in transformation frequency, up to 31.7% in the 'Jack' variety. Using CP4-EPSPS as a selectable marker resulted in higher regeneration rates and transformation frequencies compared to CG10-EPSPS. The protocol was successful across 15 elite soybean lines, with transformation frequencies ranging from 3% to 35.8%, facilitating genetic engineering and basic research in soybean.

2.2.2 *In-planta* transformation

Hu et al. (1999) Soybean is a recalcitrant species for *in vitro* manipulation. Chinese scientists developed two *in planta* non-tissue culture soybean transformation procedures: (1) via the “pollen-tube-pathway” to introduce exogenous genomic total DNA of Glycine gracilis, the seed of which consisted of 50% protein, and (2) “ovarian injection” with exogenous plasmid DNA containing atrazine-resistant gene. A high yield and high seed protein (45.44%) cultivar, ‘Heisheng 101’, resulted from the first method, and atrazine-resistant F1, F2, and F3 plants were obtained from the second method. Both exogenous single-genic Mendelian traits and multi-genic quantitative traits were transferrable with these simple and inexpensive procedures. However, some controversy exists with the acceptance of these novo procedures; mainly because instead of the standard Southern blotting, the RAPD and dot blotting techniques were used in the molecular confirmation of the transgenic status in the reported studies.

Weigel et al. (2006) reported the breakthrough in Arabidopsis research was the invention of the vacuum-infiltration method, for obtaining transformants with high efficiency and without the use of tissue culture. They described the different processes involved such as floral dip, vacuum infiltration, and spraying. They yield transformants at frequencies ranging up to several percent, with the most common frequency being 0.1%-1%.

Liu et al. (2009) improved the soybean transformation by ovarian drop by optimizing the length of the transformation path by cutting the style. Using a minimal Gus linear gene cassette as foreign DNA, a maximum transformation frequency of 11% was achieved in flowers of soybean cultivar “Liaodou 14” with the style mostly removed while only the stigma was removed, the style partially cut off, and the ovary was partially removed. Slicing yielded transform frequencies of 0%, 1%, and 2%, respectively. An average transformation frequency of 8.2% was obtained when 619 flowers from three soybean cultivars ('Liaodou 14', 'Liaodou 13' and 'Tiefeng 29') were transformed using this optimized method.

Kalbande et al. (2016) reported in their study a simple *in planta* method for efficient transformation of diploid cotton Gossypium hirsutum cv LRK-516 Anjali using *Agrobacterium* tumefaciens EHA-105 containing recombinant binary vector plasmid pBinAR with Arabidopsis At-NPR1 gene, with steps of *In -vitro* regeneration of plants that are completely avoided. Four days old Plantlets were used for transformation, the cotyledon leaves were cut vertically, bisecting the shoot tip moderately and exposing the meristem cells at the apical meristem. Transgenes were confirmed by the presence of the At-NPR1 gene with the promoter ptII gene. Using this method, a transformation frequency of 6.89% was determined for the CV LRK-516.

Hada et al. (2018) optimized *Agrobacterium* tumefaciens-mediated transformation of soybeans using half-seed explants, focusing on eight parameters. They achieved maximum transformation efficiency of 14% and regeneration efficiency of 45% using explants from mature seeds imbibed for 36 hours. Infection with A. tumefaciens strain EHA105 at OD600 of 0.8 in pH 5.4 medium containing 0.2 mM acetosyringone and 450 mg/L, L-cysteine, followed by sonication for 10 seconds, vacuum infiltration for 2 minutes, and 3-day co-cultivation on 35 mg/L kanamycin-containing medium. Transgenic lines were confirmed through assays and molecular analysis. The protocol showed high regeneration efficiency in just 76 days, offering a rapid and efficient approach for soybean genetic manipulation.

2.3. Sonication assisted *Agrobacterium* mediated transformation

Santarem et al. (1998) explored Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) for improving efficiency. They used immature soybean cotyledons as explants, evaluating various parameters' effects on transient β-glucuronidase (GUS) activity. The highest GUS expression was achieved with a 2-second sonication of cotyledons in *Agrobacterium* solution (0.11 OD600nm), followed by 3-day co-cultivation at 27°C with the explant's abaxial side in contact with the culture medium. Acetosyringone addition during co-culture enhanced transient expression. Cultivar and binary vector variations didn't affect outcomes. Moderate tissue disruption was observed with 2-second sonication, while longer durations caused more extensive damage.

Trick et al. (1998) achieved successful transformation of soybean embryogenic suspension cultures using Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT). They inoculated 10–20 embryogenic clumps with diluted log-phase *Agrobacterium* and sonicated them for 0–300 seconds. After 2 days of co-culture with acetosyringone, the medium was replaced with Timentin®-containing medium. Two weeks post-SAAT, hygromycin selection was applied, leading to the isolation of transgenic clones 6–8 weeks later. Without SAAT, hygromycin-resistant clones were not obtained. Southern hybridization confirmed T-DNA integration in transformed tissue.

2.4. *Agro*-injection based *in-planta* transformation

Zia et al. (2011) explored *in planta* and *Agro*infiltration methods for soybean transformation, bypassing tissue culture steps but with typically low efficiencies. They injected overnight-grown *Agrobacterium* tumefaciens EHA105 with pROKIILFYGUSint plasmid into soybean pods at different developmental stages. GUS histochemical analysis and PCR confirmed transformation. Notably, injecting *Agrobacterium* into pods at 2-3 days after formation (stage I) resulted in 14.2% and 6.45% transformation efficiencies for NARC-7 and NARC-4 plants, respectively. The highest GUS expression (39.16%) was observed in NARC-7 seeds when pods were treated at the late developmental stage (stage III). This study is the first to demonstrate embryo transformation via pod *Agro*-injection, offering a direct method for producing transformed seeds for further analysis at the progeny level.

Solliman et al. (2021) developed a rapid and cost-effective transient transformation assay using *in-planta Agro*infiltration in potato seeds, achieving higher efficiency than conventional methods. They used PCR to amplify a single copy of the Bacillus thuringiensis Crystal Protein Cry1Ca (cry1Ca) gene for screening. Potato slices were *Agro-injected* with plasmid pRI201-AN-GUS DNA for transient assay of the β-glucuronidase gene (GUS). Transformation was conducted using disarmed *Agrobacterium* tumefaciens strain LBA4404 harboring a binary vector pRI201-AN-GUS-Cry1Ca. Genetically modified potato plants were selected on kanamycin-containing medium and sub-cultured on medium supplemented with BA and NAA. *Agro*-injection proved efficient for transient GUS expression and gene analysis. Successful transformation of Cry1Ca gene potato plants is expected to enhance yield and food security in Saudi Arabia. The study provides a protocol for potato genetic transformation via *Agro*-injection using *A. tumefaciens* carrying the pRI201-AN-GUS-Cry1Ca plasmid, with evidence from histological and molecular assays.

2.5.1 BADH2

Arikit et al. (2010) explored the biosynthesis of 2-acetyl-1-pyrroline (2AP) in Soybean (*Glycine max*). They discovered that aromatic soybean varieties containing 2AP lacked activity of the AMADH enzyme. They characterized two genes in soybean, GmAMADH1 and GmAMADH2, akin to the rice gene Os2AP encoding AMADH. While the transcription level of GmAMADH2 was lower in aromatic varieties, the expression of GmAMADH2 remained unchanged. A double nucleotide deletion in exon 10 of GmAMADH2 was identified in all aromatic varieties, causing a frame-shift mutation and a premature stop codon. Introducing a GmAMADH2-RNAi construct into non-aromatic wild-type calli suppressed GmAMADH2 activity, inhibited AMADH synthesis, and induced 2AP biosynthesis. These findings suggest that the deficiency of GmAMADH2 product, AMADH, plays a similar role in soybean as in rice by promoting 2AP biosynthesis, indicating a conserved mechanism among different plant species.

Juwattanasomran et al. (2010) identified an association between a single nucleotide polymorphism (SNP) in exon 10 of the GmBADH2 gene and fragrance in the vegetable soybean cultivar Kaori (Glycine max (L.) Merr.). This SNP led to an amino acid substitution in a highly conserved motif of the GmBADH2 protein, affecting its functional activity.

In their study, Juwattanasomran et al. (2010) sequenced GmBADH2 in Chamame, a fragrant soybean cultivar, and identified a new fragrance allele characterized by a 2-bp (TT) deletion in exon 10. This deletion caused a reading frame shift and introduced a premature stop codon, resulting in the inhibition of protein function and the development of fragrance. The old and new fragrance-promoting alleles were designated as GmBADH2-1 and GmBADH2-2, respectively. They developed a functional marker to genotype GmBADH2-2, capable of distinguishing fragrant and non-fragrant soybeans and differentiating between different fragrant soybeans. Through QTL mapping using an F2 population and Chamame as the fragrance donor, they found that the fragrance QTL and the functional marker were closely located, confirming the association between GmBADH2 and fragrance.

In their study, Juwattanasomran et al. (2011) investigated fragrance in soybeans, attributed to 2-acetyl-1-pyrroline (2AP). They focused on the BADH2 gene, associated with fragrance in rice. Using an RIL population from fragrant cultivar "Kaori" and non-fragrant cultivar "Chiang Mai 60" (CM60), they found STS markers from BADH2 homologs linked to 2AP production. Genetic mapping revealed the QTL position of fragrance coincided with GmBADH2. Sequence analysis showed a SNP in exon 10 of GmBADH2, resulting in an amino acid change crucial for its function. They developed five SNP markers for fragrance, aiding marker-assisted breeding of fragrant soybeans.

In their study, Juwattanasomran et al. (2012) confirmed an association between fragrance in soybeans and a single nucleotide polymorphism (SNP) in exon 10 of the GmBADH2 gene. They identified a new fragrance allele in the cultivar Chamame, characterized by a 2-bp (TT) deletion in exon 10, leading to a premature stop codon and fragrance development. They designated the old and new fragrance-promoting alleles as Gmbadh2-1 and Gmbadh2-2, respectively. They developed a simple and co-dominant functional marker for genotyping Gmbadh2-2, useful for distinguishing fragrant and non-fragrant soybeans and differentiating fragrant varieties. QTL mapping confirmed the association between GmBADH2 and fragrance in Chamame.

In their study, Qian et al. (2022) confirmed that the aroma in specific soybean cultivars is due to the presence of 2-acetyl-1-pyrroline (2AP), a potent volatile compound previously identified in soybeans. Mutations in GmBADH2 were found in aromatic cultivars Quxian No. 1, Xiangdou, and ZK1754, including 10 SNPs, an 11-nucleotide deletion in exon 1, and a 2-base pair deletion in exon 10, resulting in frame shifts and premature stop codons. Genetic analysis suggested a single recessive gene inheritance pattern for the aromatic trait, implicating the mutated GmBADH2 gene.

qRT-PCR and CRISPR/Cas9 experiments confirmed the expression and function of GmBADH2. A functional marker based on the mutated GmBADH2 sequence was validated in an F2 population, showing perfect association with aroma phenotypes. These findings shed light on the molecular basis of 2AP formation in soybeans and offer potential for marker-assisted breeding of aromatic vegetable soybean cultivars.

Chen et al. (2024) investigated the function of betaine aldehyde dehydrogenase (BADH) genes in tobacco (*Nicotiana tabacum* L.) concerning 2-acetyl-1-pyrroline (2AP) accumulation, a key fragrance compound. They identified four BADH genes in tobacco, with two resembling wolfberry BADH and named NtBADH1a and NtBADH1b, and two resembling tomato AMADH2, named NtBADH2a and NtBADH2b. Expression analysis revealed distinct functions for NtBADH1 and NtBADH2 genes. Using CRISPR/Cas9, they generated single and double mutants for each gene, resulting in frameshift mutations preventing protein accumulation. Notably, only the double mutant Ntbadh2a-Ntbadh2b exhibited a popcorn-like scent and 2AP accumulation, indicating that simultaneous inactivation of NtBADH2a and NtBADH2b is necessary for 2AP production, not associated with NtBADH1.

2.5.2 KTI

Jofuku et al. (1989) investigated a mutation in the soybean Kunitz trypsin inhibitor (KTi) gene that inhibits the accumulation of KTi protein during seed development. They found a significant reduction in KTi3 mRNA levels in mutant embryos compared to normal ones. Sequencing revealed three nucleotide differences in the KTi3 gene between normal and mutant lines, causing a frameshift mutation and premature termination of translation. This frameshift mutation destabilizes KTi3 mRNA, leading to a sharp decrease in its prevalence.

Pesic et al. (2007) examined the relationship between two key trypsin inhibitors (TI) in soybean, Kunitz (KTI) and Bowman-Birk (BBI), and their corresponding trypsin inhibitor activity (TIA). They analyzed twelve soybean genotypes and found significant differences in TI levels and TIA. A strong positive correlation was observed between KTI and total BBI levels ($r = 0.94$, $P < 0.05$), but no correlation was found between TI levels and TIA. These findings suggest a connection between major TI levels in soybean, which could aid in improving the health impacts of soy proteins by understanding their relationship with trypsin inhibitor activity.

Liener (1994) discussed several components in soybeans that negatively affect the nutritional quality of the protein. Heat treatment destroys protease inhibitors and lectins, which can cause pancreatic hypertrophy/hyperplasia and interfere with nutrient absorption, respectively, leading to growth inhibition. Other heat-stable factors like goitrogens, tannins, phytoestrogens, oligosaccharides, phytate, and saponins also have antinutritional effects. Processing soybeans under severe alkaline conditions can lead to lysinoalanine formation, damaging rat kidneys, but this is less common with milder alkaline conditions. Allergic responses in humans, as well as calves and piglets, may occur with dietary exposure to soybeans.

In their study, Wang et al. (2023) addressed the challenge of breeding soybean cultivars with low trypsin inhibitor (TI) content, a key anti-nutritional factor in soybean meal affecting digestibility. They identified two seed-specific TI genes, Kunitz trypsin inhibitor 1 (KTI1) and KTI3, and generated mutant alleles using CRISPR/Cas9 in *Glycine max* cv. Williams 82 (WM82). The kti1/3 mutants exhibited significantly reduced KTI content and TI activity compared to WM82 seeds without affecting plant growth or maturity. A T1 line, #5-26, carrying double homozygous kti1/3 mutant alleles was identified, and markers were developed to co-select for these mutant alleles. These findings offer a promising approach to accelerate the development of elite soybean cultivars with low TI traits.

Kim et al. (2024) in their study they tackled the challenge of reducing protease inhibitor content in soybean seeds, which limits their direct inclusion in animal feed. They employed CRISPR/Cas9 gene editing to create mutations in Bowman–Birk protease inhibitor (BBi) genes, aiming to lower inhibitor levels without the need for energy-intensive heat treatment. Through *Agrobacterium*-mediated transformation, they generated stable transgenic soybean events harboring mutations in BBi genes. Analysis via Sanger sequencing, proteomics, inhibitor activity assays, and qRT-PCR revealed significant reductions in both trypsin and chymotrypsin inhibitor activities in mutant lines compared to wild-type plants, establishing an allelic series of loss-of-function mutations in BBi genes.

CHAPTER III

MATERIAL AND METHODS

The present investigation entitled "Assessment of critical parameter associated with *in planta* transformation of elite soybean genotype(s) (*Glycine max* L.)" was carried out at Biotechnology Centre, Department of Agricultural Botany, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth Akola. The details of the material and methods used in the present investigation are given below.

3.1 Material

3.1.1 Genotypes

The experimental material of the present investigation comprised one vegetable-type Swarna Vasundhara(SV), and two grain-type varieties Swarna soya (AMS MB 5-18) and PDKV yellow gold (AMS 1001). Swarna soya was obtained from ICAR, RCER, Ranchi, Jharkhand and grain type varieties were obtained from the Central Research Unit, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola. The specific characters of the varieties are summarized in Table 3.1.

Table 3.1. Soybean genotypes used in the present investigation

SN	Genotype	Released By	Salient Features
1.	Swarna Vasundhara	ICAR-RCER, Ranchi	Vegetable Type
2.	(AMS 100-39) PDKV Amba	DR. PDKV, AKOLA	Pod shattering resistance, moderately resistant to girdle beetle and stem fly and charcoal rot disease
3.	AMS-1001 (PDKV Yellow Gold)	DR. PDKV, AKOLA	Grain Type, Charcoal rot and YMV disease resistant, High Yielding.

3.1.2 *Agrobacterium* vector for plant transformation

In the current experiment, the *Agrobacterium tumefaciens* EHA 105 strain, harboring the plasmid vector pDIRECT_22A, was used for the genetic transformation of soybean. The plasmid vector, pDIRECT_22A, contains guide RNAs (gRNAs) sequence specifically targeting the GmBADH2 and KTI genes. Figures 3.1 and 3.2 illustrate the partial construct map, highlighting the T-DNA region, which carries theCas9 sequence and respective promotors and gRNAs designed for precise editing of the GmBADH2 and KTI genes. The pDIRECT_22A plasmid was introduced into the EHA 105 strain of *Agrobacterium tumefaciens* via the freeze-thaw method as described by Weigel et al. (2006).Two transformation approaches were utilized: Sonication-Assisted *Agrobacterium*-Mediated Transformation (SAAT) and an *Agro*-injection based *in planta* transformation system.

3.1.3 Equipment

Equipment's like for the current study comprises LAF (Laminar Air Flow) cabinet, Shaker Incubator, Cooling centrifuge, Electrophoresis unit, PCR, Gel electrophoresis etc.

3.2 Methodology

3.2.1 *Agrobacterium tumefaciens* culture preparation

The *Agrobacterium* strain EHA 105 carrying the pDIRECT_22A plasmid vector, which harbors sgRNA targeting the BADH2/KTI genes, will be maintained on solid LB medium supplemented with rifampicin (30 mg/l) and kanamycin (50 mg/l). The culture will be revived every 30 days on fresh medium, incubated at 28±2°C for 48 hours, and then preserved at 4°C.

For the preparation of *Agrobacterium* inoculum, a single colony from a freshly grown plate was inoculated into 5 mL of Luria Broth (LB) supplemented with the respective antibiotics and incubated overnight at 28±2°C, under orbital shaker with 150 rpm. The following day, 100 µl of the overnight-grown culture was transferred into 50 ml of LB broth

Fig 3.1: Partial map of T-DNA of vector p_DIRECT_22A having gRNA to target GmBADH2 gene

Fig 3.2: Partial map of T-DNA of vector p_DIRECT_22A having gRNA to target GmKTI gene.

supplemented with 50 µl of acetosyringone stock solution (40 mg/ml) in a 50 ml culture and the inoculum density was adjusted at (0.6) OD_{600}nm.

3.2.2 Preparation of antibiotics

3.2.2.1 Preparation of rifampicin stock (30 mg/ml)

STEP 1 150mg of rifampicin dissolved in DMSO (about 1 to 1.5 ml) and volume make up to 5 ml with sterile double distilled water.

STEP 2 Filter through a 0.45 µm syringe filter.

STEP 3 Aliquots were prepared, labeled properly, and stored at -20°C for further use (repeated freezing and thawing of rifampicin stock should be avoided).

3.2.2.2 Preparation of kanamycin stock (100mg/ml)

STEP 1 0.5g of kanamycin dissolved in 5 ml double distilled water.

STEP 2 Filtered through 0.45 µl syringe filter.

STEP 3 An aliquot was prepared, labeled properly, and stored at -20°C for further use.

3.2.2.3 Preparation of Acetosyringone stock (40 mg/ml)

STEP 1 200mg of Acetosyringone dissolved in DMSO (about 1 to 1.5 ml) and volume make up to 5 ml with double distilled water.

STEP 2 Filter through a 0.45 µm syringe filter.

STEP 3 Aliquots were prepared, labeled properly, and stored at -20°C for further use (repeated freezing and thawing of Acetosyringone stock should be avoided).

3.2.3 Sonication of full seed

Soybean seeds of the respective genotypes were imbibed in distilled water for 12 hours and subsequently surface sterilized using Tween20. The *Agrobacterium* culture was prepared according to the procedure described in Section 3.2.1. Prior to infection, 50 µl of acetosyringone stock solution was added to 50 mL of *Agrobacterium* culture and incubated for 30 minutes. The entire batch of imbibed seeds was subjected to treatment by immersing in the prepared *Agrobacterium* culture, and sonication according to the treatment (Table 3.2) with time intervals (control, 15 sec, 30 sec, 45 sec). Then seeds were directly sown in pots containing autoclave-sterilized soil and maintained in a transgenic greenhouse.

3.2.4 Sonication of imbibed half seed

For half seed sonication treatment soybean seeds from each genotype were first soaked in distilled water for 12 hours. Once soaked, they were surface sterilized with Tween® 20 to remove any potential contaminants. The *Agrobacterium* culture was prepared as described in Section 3.2.1, with 50 µl of acetosyringone from the stock added to 50 ml of culture and left to sit for 30 minutes to boost infection efficiency. After sterilization, the seeds were carefully cut into halves with a sterile scalpel. The half-seeds were then completely immersed in the *Agrobacterium* culture and subjected to different sonication times: no sonication (control), 15 seconds, 30 seconds, and 45 seconds. Once the sonication treatment was done, the seeds were immediately planted in pots filled with autoclave-sterilized soil. These pots were then placed in a transgenic greenhouse, where they were kept under carefully controlled conditions to support their growth and potential transformation.

3.2.5 ***Agro*-injection at exposed embryo in half seed**

For the *Agro*-injection treatment, soybean seeds and the *Agrobacterium* culture were prepared using the same procedure as mentioned earlier. The following day, the seeds were cut in half using a sterile scalpel and blade, ensuring the embryo remained intact. The *Agrobacterium* culture, adjusted to an optical density (OD) of 0.6, was loaded into insulin syringes. The embryo of each half-seed was then punctured at designated sites, depending on the treatment. For Teatment-1(T1), injections were performed without *Agrobacterium*, serving as the control. For Teatment-2(T2), the punchers were made at the epicotyl portion of the embryo. For T3, injections were done at two sites: the epicotyl portion and the neck between the epicotyl and hypocotyl of the embryo and for T4 *Agro*-injection was given at 3 different sites. After the injections, the seeds were briefly dried on tissue paper before being sown directly into pots containing autoclave-sterilized soil. These pots were then placed in a transgenic greenhouse and maintained under controlled conditions to facilitate plant growth and transformation.

3.2.6 *Agro*-injection at florets

For the *Agro*-injection treatment at the floret stage, the procedure begins by selecting flower buds that are expected to open the following day. Using micro-needles, the *Agrobacterium* culture is carefully loaded and precisely injected into the ovary region of the selected buds. This delicate process targets the reproductive structures, ensuring optimal conditions for transformation. The treated buds are then monitored under transgenic greenhouse conditions to evaluate the efficiency and success of the transformation.

3.2.7 Assessment of parameter(s) responsible for Pod Bearing% and rate of CRISPR/Cas9 component delivery

The critical parameters studied were the pod-bearing percentage and the efficiency of CRISPR/Cas9 component delivery to evaluate the effectiveness of the *in-planta* transformation method.

$$\text{Pod Bearing\%} = \frac{\text{No. of pod developed} \quad \text{x } 100}{\text{Total no. of flowers}}$$

$$\text{Rate of CRISPR\textbackslash Cas9 delivery} = \frac{\text{No. of individual positive for CRISPR\textbackslash Cas9}}{\text{Total No. of individual planted}}$$

Table 3.2 Treatments of *Agro*-injection and Sonication using half and full seed in Soybean

SN	Method	Treatments
1	*Agro*-injection at exposed embryo in half seed	T1-Injection without *Agrobacterium.*
		T2-1st site- at epicotyl portion of embryo
		T3-1st + 2nd site- at epicotyl portion + neck of epicotyl and hypocotyl of embryo
		T4-1st + 2nd site + 3rd site- at epicotyl portion + neck of epicotyl and hypocotyl + hypocotyl of embryo
2	Sonication of	T1-Sonication of imbibed seed in water

	imbibed full seed	T2-15 Sec
		T3-30 Sec
		T4-45 Sec
3	Sonication of imbibed half seed	T1-Sonication of imbibed seed in water
		T2-15 Sec
		T3-30 Sec
		T4-45 Sec

*****For each treatment *Agro*-injection of 0.6OD culture at florets were given

3.2.8 Plasmid isolation

Table3.3 : Composition of different solutions required for plasmid isolation

Solution	Chemical component	Concentration
1. Alkaline lysis solution I	Glucose	50mM
	Tris HCL(pH 8.0)	25mM
	EDTA(pH 8.0)	10mM
2. Alkaline lysis Solution-II	NaOH	0.2N
	SDS	1%
3. Alkaline lysis solution-III (pH 4.8)	5M potassium acetate	60 ml
	Glacial acetic acid	11.5 ml
	Water	28.5 ml

3.2.9 Isolation of Plasmid DNA from transformed *Agrobacterium* cells

The plasmid DNA was isolated using the alkali lysis method of giving by (Warburton, 2005) with minor modifications.

Note:

1. All purification steps were carried out on the ice
2. 5 ml of overnight grown *Agrobacterium* culture in LB media was used
3. Didn’t vertex the solution after addition of lysis solution II
4. The lysis reaction was carried out within 5 minutes

3.2.10 Procedure for isolation of plasmid DNA using Alkaline Lysis methods

STEP 1 A single colony was picked from a freshly streaked selective plate and inoculated with 5 ml of LB medium containing 50mg/l kanamycin. The bacterial culture was harvested by centrifugation at 8000rpm in a micro-centrifuge for 2 minutes at 4°C. The supernatant was aspirated to remove all remaining medium.

STEP 2 Bacterial pellet was re-suspended in ice cold alkaline lysis solution-I(100 µl).Dissolved suspension was transferred to a micro centrifuge tube and vortexes until no cell clumps remained.

STEP 3 Lysis Solution-II (200 µl) was added and mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly clear.

STEP 4 Alkaline solution III (150 µl) was added and mixed immediately and gently by inverting the tube 4-6 times to avoid localized precipitation of bacterial cell debris and left tubes on ice for 3-5minutes.

STEP 5 Centrifuged for 5 min to pellet cell debris and chromosomal DNA. The supernatant was transferred to fresh tubes, by decanting or pipetting without disturbing the white precipitate

STEP 6 Equal volumes of phenol: chloroform: isoamyl alcohol was added, organic and aqueous phases were vortexed and then centrifuged at maximum speed for 2 minutes at 4°C in a micro centrifuge. The upper aqueous layer was transferred to a fresh tube.

STEP 7 Nucleic acid was precipitated by adding 2 volumes of ethanol at room temperature. The solution was mixed by vortexing and allowing the mixture to stand at room temperature for 2 minutes.

STEP 8 The nucleic acid was precipitated by centrifugation at maximum speed for 5 minutes at 4°C in a microfuge.

STEP 9 Remove the pellet by gentle aspiration, then pellet was washed with 70% ethanol.

STEP 10 Ethanol was completely evaporated by keeping tubes open at room temperature for 5-10 minutes.

STEP 11 Finally, the nucleic acid was dissolved in 50 µl of TE buffer containing 20µg/ml DNase free RNaseA. The solution was vortexed for a few seconds, and stored at -20°C.

3.2.11 Confirmation of Ti plasmid by PCR amplification

Plasmid extracted from the bacterial culture was used as template DNA. The PCR reaction was carried out using the sequence-specific marker for BADH2/KTI and Cas9.

3.3 Molecular screening of putative transformants

3.3.1 DNA isolation

STEP 1 Collected the clean leaf samples without midrib in 2 ml Eppendorf centrifuge tubes.

STEP 2 Grinded leaf tissue in liquid nitrogen with micro pestles.

STEP 3 Powdered leaf tissue was homogenate by adding 1 ml of CTAB extraction buffer and mixed it by inverting for 5 min.

STEP 4 The tubes containing homogenate were incubated in a water bath at 65^0C for 45 min.

STEP 5 After Incubation, the tubes were centrifuged at 13,000 rpm for 10 min.

STEP 6 After that supernatant was collected and transferred to another 1.5 ml centrifuge tube and 0.8ml of Chloroform: Isoamyl alcohol (24:1) added and mixed properly by inverting for 5 min.

STEP 7 The samples were centrifuged at 13,000 rpm for 10 min. The aqueous layer was collected in another 1.5 ml centrifuge tube.

STEP 8 The above steps were repeated (STEP 6 and STEP 7) and final supernatants were collected.

STEP 9 Upper layer was mixed with double volume of CTAB precipitation solution and samples were incubated at room temperature for 1h.

STEP 10 After incubation, the tubes were centrifuged at 13,000 rpm for 10 min.

STEP 11 Supernatant were discarded and precipitation dissolved in 0.7 ml of 1.2 M NaCl solution.

STEP 12 0.7 ml of chloroform were added to each tube and mixed properly by inverting for 5 min.

STEP 13 The tubes were centrifuged at 13,000 rpm for 10 min and supernatants were collected into 1.5 ml centrifuge tubes.

STEP 14 0.6 volume of iso-propanol were added to each sample and incubate at room temperature for 10 min.

STEP 15 After incubation, the tubes were centrifuged at 13,000 rpm for 10 min and pellets were collected by discarding supernatant.

STEP 16 Pellet washed with 0.1 ml of 70% ethanol followed by centrifugation at 13,000 rpm for 5 min.

STEP 17 Supernatant was discarded, pellet was air dried and dissolved in 20 µl of nuclease free water.

3.3.2 Genomic DNA extraction buffer

1. 1M Tris-HCl solution (pH 8)

121.1gm Tris-HCl was dissolved in 800 ml distilled water. Volume was made up to 1000 ml with distilled water and was sterilized by autoclaving after adjusting the pH to 8 with HCl.

2. 0.5M EDTA solution (pH 8.0)

186.1 gm EDTA was dissolved in 800ml distilled water. The solution was stirred vigorously on a magnetic stirrer and adjusted to pH to 8.0 by adding NaOH pellets. The final volume was made up to 1000 ml with distilled water followed by sterilizing in autoclaving**.**

3. 5M NaCl

Dissolved 73.05gm NaCl in 200ml distilled water and made final volume up to 250ml with distilled water. (Warm water was used).

3. Chloroform

4. Ethanol (70%)

5. Isoamyl alcohol

7. Isopropanol

Table 3.4: Composition of CTAB buffer

Chemical	Stock Concentration	Working concentration	Volume requires per 1000ml
Tris-HCl (pH 8)	1M	0.1	100
NaCl	5M	1.4 M	280
EDTA (pH 8)	0.5M	20mM	40

Make up the volume with autoclaved distilled water

3.3.3 PCR amplification of putative transformants

The putative mutants were screened using a gRNA-specific marker/primer for BADH2/KTI and Cas9 sequence-specific marker.

Table3.5: Primer sequence used for screening of putative transformants

Gene	Direction	Primer sequence	band size
GmBADH2 gRNA	Forward	GTCTATGAATAACTGCCGAT	425 bp
	Reverse	CGAACGGATAAACCTTTTCACG	
KTI gRNA	Forward	GATTGCTGCACGCGATACAGTAGA	425 bp
	Reverse	CGAACGGATAAACCTTTTCACG	
Cas9	Forward	GGAACTTCGAAGAGGTGGTG	810 bp
	Reverse	CTTCACAGTCTGGAGGATTC	

The PCR was performed according to the PCR profile given below

Table 3.6 Temperature profile for amplification of gRNA specific primers

Sr. No.	Step	Temp	Time	Cycle
1	Initial denaturation	94^oC	5 min	1
2	Denaturation	94^oC	30 sec	32
3	Annealing	48^oC	30 sec	
4	Extension	72^oC	45 sec	
5	Final Extension	72^oC	10 min	
6	Hold	04^oC	∞	

Table 3.7 PCR Temperature Profile for Amplification of gRNA-Specific and Cas9-Specific Primers

SN	Step	Temp	Time	Cycle
1	Initial denaturation	94^oC	5 min	1
2	Denaturation	94^oC	30 sec	32
3	Annealing	54^oC	30 sec	
4	Extension	72^oC	45 sec	
5	Final Extension	72^oC	10 min	
6	Hold	4^oC	∞	

The reaction mixture was prepared in 0.2 ml thin-walled PCR tubes containing the following components. The total volume of each reaction mixture was 10 µl.

Table 3.8 PCR components and their volumes in a reaction mixture

SN	Components	Concentration	Volume / 10 µl
1	DNA	30-40 ng/ µl	1.0 µl
2	Forward primer	10pmol	0.5 µl
3	Reverse primer	10pmol	0.5 µl
4	NFW (Nuclease free water)	-	3.0 µl
5	Master Mix	2X	5.0µl
	Total		**10 µl**

After completion of the required cycles of PCR reaction, the samples were stored at -20^{o}C in a refrigerator until further use. Products of PCR amplification of the plasmid was compared and analyzed on the 2.0% agarose gel.

3.3.4 Agarose gel electrophoresis

STEP 1 The PCR products were confirmed on 2.0% and DNA and plasmid were confirmed in 0.8 and 1.2% agarose gel electrophoresis respectively. The detailed procedure is given below.

STEP 2 A sufficient 1X electrophoresis buffer was prepared from 50X stock.

STEP 3 Add Agarose powder 2.0%,1.2%,0.8% for PCR product, plasmid and DNA to TAE buffer (1X) and was dissolved by melting at 100°C. The solution was cooled to 50°C and ethidium bromide was added (0.5ug/ml) and the comb was positioned at 0.5 -1.0 mm above the plate.

STEP 4 Then the melted agarose solution was poured into the gel tray and was allowed to polymerize. The gel tank was filled with TAE buffer (1X) just enough to cover the surface of the gel to a depth of 1 mm.

STEP 5 The DNA sample was mixed with gel loading buffer and it was slowly loaded into the wells of the submerged gel using disposable micro tips.1 kb DNA ladder was used as a molecular weight marker.

STEP 6 The system was connected to the power supply and electrophoresis was carried out at 80 volts for 30-45 min.

STEP 7 Gel was examined by a gel documentation system

3.4 OBSERVATIONS RECORDED

To fulfil the objective of the study, following observations were recorded for all treatments.

Table 3.9. Recorded observations

Treatment	Observations
Agro-injection at exposed in half-seed Sonication of imbibed full seed Sonication of imbibed half seed	1. Days to emergence 2. Germination percentage 3. Days to 1^{st} flower 4. Days to 50% flowering 5. Days to harvesting 6. No. of seed/pod 7. Seed size 8. Pod size 9. Total No. of T0 seeds developed 10. PCR-Positive plant

3.5 Statistical analysis

Three replications were sown for each treatment and genotype in combination, along with a control. The three methods were used viz. sonication of full seed, sonication of half-seed, and *Agro*-injection at different sites of the embryo each having 4 treatments. Observations were recorded for each treatment in three replicates."

The data of the present investigation was analyzed and ANOVA was carried out by using a factorial completely randomized design. The procedure is given by Panse and Sukhatme (1988).

The F test was used to test the significance.

$\overline{\mathrm{X}} = \Sigma \mathrm{X_i} / \mathrm{n}$

Where,

X = Mean

$\Sigma \mathrm{X_i}$ = Sum Of i^{th} observation

N = Total number of observations

SD = $\sqrt{\sum (X_i - x)^2} \, / \, n$

Where,

SE = Standard error

SD = Standard deviation

3.6 Place and duration of experiment

Research work was conducted during the academic year 2021-23 at the Biotechnology Centre, Department of Agricultural Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

CHAPTER IV

RESULT AND DISCUSSION

The present investigation, titled "Assessment of critical parameter associated with *in planta* transformation of elite soybean genotype(s) (*Glycine max* L.)" aimed to evaluate the efficacy of two *in-planta* transformation methods Sonication Assisted *Agrobacterium* mediated transformation and *Agro*-injection along with three soybean genotypes one vegetable-type Swarna Vasundhara (SV), and two grain-type varieties Swarna soya (AMS MB 5-18) and PDKV yellow gold (AMS 1001). The objective was to determine the superior method and to assess key transformation parameters associated with *in-planta* methods.

In-planta transformation is an *in-vivo* method for *Agrobacterium* mediated genetic transformation that circumvents the need for tissue culture conditions. Carried out using *Agrobacterium tumefaciens* strain EHA 105, transformed with the pDIRECT_22A plasmid harboring GmBADH2/KTI sgRNA.

For *in-planta* transformation, both the embryo and ovary were selected as target organs. When the embryo was targeted for *in-planta* transformation, the following treatments were applied: 1) Full seed sonication, 2) Half seed sonication, and 3) *Agro*-injection at the embryo in half seed. The second target organ for *in-planta* transformation was the ovary. To target the ovary, flowers were selected based on the time of pollination, with particular focus on flowers approximately 8 hours after pollination. The ovary was then targeted using a very fine needle. Seeds that were set from the *in-planta* experiment were carefully tagged, and observations were recorded on a weekly basis.

In parallel, additional *in-planta* transformation attempts were conducted under controlled conditions during this period. The results obtained from the present investigation are presented and discussed in the following sub-headings, organized according to the study objectives. The findings are compared with relevant previous studies to draw well-supported and conclusive interpretations.

4.1 Confirmation of plasmid harboring SgRNA of BADH2/KTI Gene

The *Agrobacterium tumefaciens* strain EHA 105 was transformed with the pDIRECT_22A plasmid harbouring GmBADH2/GmKTIsgRNAs using the freeze-thaw method as described by Holsters et al., (1978), with minor modifications. The transformed strain of EHA 105 carrying the pDIRECT_22A plasmid, which contains sgRNAs targeting the BADH2/KTI genes, was cultured on solid LB medium supplemented with rifampicin (30 mg/L) and kanamycin (50 mg/L). Positive colonies were selected from the transformed plates for further analysis. Plasmid DNA was isolated from the recombinant strains and confirmed by colony PCR using sgRNA-specific primers targeting the BADH2/KTI genes. Successful transformation was verified by PCR amplification of the insert sequence, with the expected 425 bp product size for both target genes (Plate 4.1E).

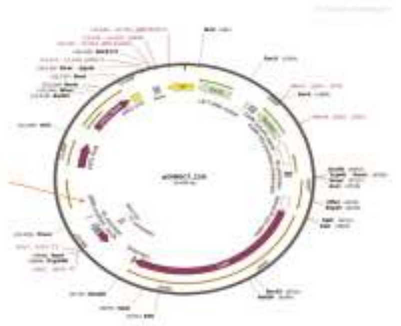
4.2 Full-Seed sonication: an *In-Planta* method

Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) has proven to be an effective method for transforming soybean and various plant species. This study aimed to assess the impact of different sonication time intervals on transformation efficiency and genotype-specific responses in soybean. Whole soybean seeds were used as target tissue, soaked in distilled water for 12 hours, and surface-sterilized. The seeds were then immersed in *Agrobacterium* culture (O.D. 0.6) and subjected to sonication for 15, 30, or 45 seconds, with a control group included for comparison. After treatment, the seeds were directly sown into pots containing sterilized soil and maintained in a transgenic greenhouse.

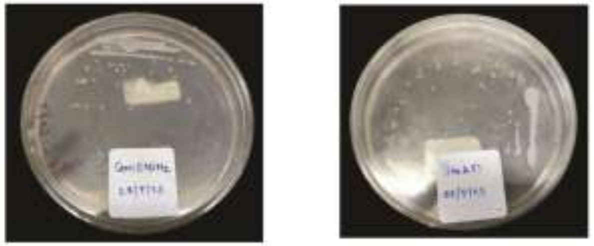
In this experiment, a total of 360 seeds were treated and planted, with 120 seeds allocated to each of the three genotypes: PDKV Amba, Suvarna Vasundhara, and PDKV Yellow Gold. For each genotype, 60 seeds were targeted for the BADH2 gene, and 60 seeds for the KTI gene. The seeds were sown in groups of five per pot, with three replicates for each of the four treatments: T1 (Control), T2 (15 Sec), T3 (30 Sec), and T4 (45 Sec).

4.1 A) Partial map of T-DNA of vector p_DIRECT_22A containing gRNA to target GmBADH2 gene;

4.1 B) Partial map of T-DNA of vector p_DIRECT_22A containing gRNA to target GmKTI gene .



4.1 D) p_DIRECT_22A plasmid harbouring BADH2/KTI gRNA



4.1 C) Transformed *Agrobacterium* EAH 105 strain with BADH2/KTI gene

L-Ladder 100bp,1-5 PCR positive *Agrobacterium* plasmid for gRNA specific primer BADH2 gene, 6-10 5 PCR 5 positive Agrobacterium plasmid for gRNA specific primer KTI gene

4.1 E) Plasmid confirmation using sgRNA specific primer

Plate 4.1: Confirmation of recombinant strain by colony PCR using sgRNA-specific primers targeting the BADH2/KTI genes

4.2.1 Effect of Full-Seed sonication treatment on germination

The germination rates of three soybean genotypes (G1: PDKV Amba, G2: Suvarna Vasundhara, and G3: PDKV Yellow Gold) were evaluated under four treatments: T1 (Control), T2 (15 Sec), T3 (30 Sec), and T4 (45 Sec) (Table 4.1). Both the control and 15-second treatments exhibited high germination rates, with G3 (95.15%) and G1 (93.45%) performing the best. However, germination rates significantly decreased in the 30-second and 45-second treatments, particularly for G2, where germination dropped to 43.29% in T4. These results suggest that prolonged sonication negatively affects germination, with the 15-second treatment being optimal for all genotypes[Table 4.1; Fig 4.1].

A similar study by Trick et al. (1998) utilized Sonication-Assisted *Agrobacterium*-Mediated Transformation (SAAT) in soybean to examine the effects of sonication duration on cotyledons, with sonication times ranging from 0.1 to 10 seconds. The reduction in germination observed in their study, alongside the enhanced transient GUS expression, was consistent across different genotypes, including the tested cultivars ('Jack', 'Chapman', and 'Kunitz').

Table4.1. Effect of full-seed sonication treatments on seed germination in experimental soybean genotypes

Treatments	Germination percentage		
	(G1)PDKV Amba	(G2)Suvarna Vasundhara	(G3)PDKV Yellow gold
T1(Control)	92	82.89	95.15
T2(15 Sec)	93.45	87.45	85.11
T3(30 Sec)	77.11	63.67	72.11
T4(45 Sec)	63.63	43.29	41.33
CV			1.25
Source	**SE(M))±**	**CD5%**	
Factor A	0.93	2.73	
Factor B	0.81	2.36	
Factor AB	1.62	4.75	

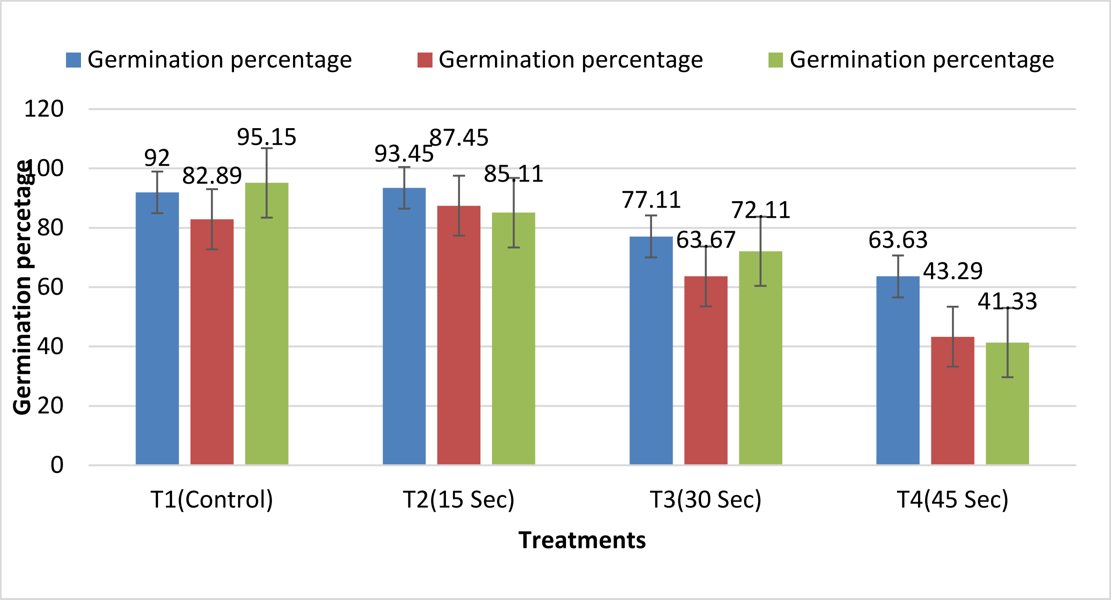


Fig 4.1. Effect of full-seed sonication treatment on seed germination in soybean genotypes

4.2 Half-Seed sonication: an *In-Planta* method

Soybean seeds from each genotype were initially soaked in distilled water for 12 hours. After soaking, the seeds were carefully cut in half using a sterile scalpel. The *Agrobacterium* culture was then prepared, and the half-seeds were fully immersed in the culture. The seeds were subjected to different sonication times: no sonication (control), 15 seconds, 30 seconds, and 45 seconds. Once the sonication treatment was completed, the seeds were immediately planted in pots containing autoclave-sterilized soil.

In the half-seed sonication experiment, a total of 360 seeds were treated and planted in pots, with 120 seeds allocated to each genotype: PDKV Amba, Suvarna Vasundhara, and PDKV Yellow Gold. For each genotype, 60 seeds were used for targeting the BADH2 gene and 60 for the KTI gene. The seeds were sown in sets of five per pot, with three replicates for each of the four treatments: T1 (Control), T2 (15 Sec), T3 (30 Sec), and T4 (45 Sec).

4.2.1 Effect of Half-Seed treatment on germination

The germination of three soybean genotypes (PDKV Amba, Suvarna Vasundhara, and PDKV Yellow Gold) was evaluated under four sonication treatments: T1 (Control), T2 (15 Sec), T3 (30 Sec), and T4 (45 Sec) (Table

4.2). The control group exhibited the highest germination rates, with PDKV Yellow Gold leading at 90.85%. A slight decline in germination was observed in T2, while a more pronounced decrease occurred in T3, particularly for Suvarna Vasundhara, which showed a germination rate of 57.78%. The lowest germination was recorded in T4, with Suvarna Vasundhara at 37.78%. These results suggest that extended sonication time negatively impacts germination, with the 15-second treatment proving to be the most effective.

In a similar study conducted by Santarem et al. (1998), it was reported that Sonication-Assisted *Agrobacterium* Transformation (SAAT) significantly enhances the efficiency of *Agrobacterium* infection by inducing a large number of micro-wounds in the target plant tissue. Using immature soybean cotyledons as explants, they evaluated various factors, including cultivar type, *Agrobacterium* optical density during infection, and sonication treatment duration. Their study also assessed the extent of tissue disruption caused by sonication and the survival rate of the explants. They found that cotyledons subjected to 2 seconds of sonication exhibited moderate tissue disruption, whereas longer sonication treatments resulted in more extensive damage and lower survival rates[Table 4.2; Fig 4.2].

Table 4.2. Effect of Half-Seed sonication treatments on seed germination in different soybean genotypes

Treatments	Genotypes		
	PDKV Amba	**Suvarna Vasundhara**	**PDKV Yellow gold**
T1(Control)	89.67	83.22	90.85
T2(15 Sec)	83.78	77	80
T3(30 Sec)	76.28	57.78	75.56
T4(45 Sec)	57.78	37.78	53.34
CV			1.92
Source	SE(M))±	CD5%	
Factor A	1.38	4.04	
Factor B	1.19	3.5	
Factor AB	2.39	7	

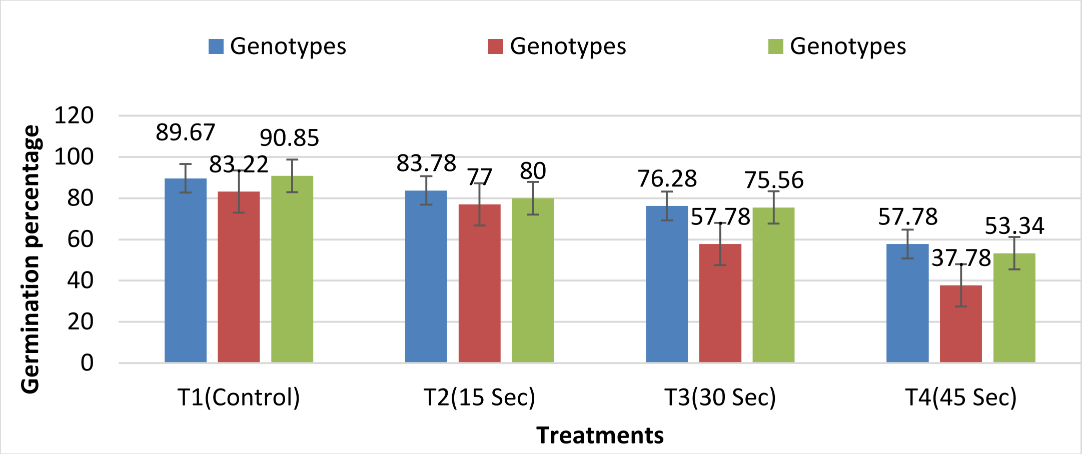


Fig4.2. Effect of Half-seed sonication treatments on seed germination in different soybean genotypes

4.3 *Agro*-injection at embryo: an *In-Planta* method

The *Agro*-injection *in-planta* method has been successfully utilized in soybean by Zia et al. (2011) and in several other crops, such as cotton, by Gurusaravanan et al. (2018). In this study, the procedure for *Agro*-injection followed the same protocol as described in section 4.2.1, where soybean seeds and *Agrobacterium* cultures were prepared. The seeds were then cut in half using a sterile scalpel and blade, ensuring the embryo remained intact. The *Agrobacterium* culture was adjusted to an optical density (OD) of 0.6 and loaded into insulin syringes. Following this, the embryo of each half-seed was punctured at specific sites according to the treatment. After the injections, the seeds were briefly dried on sterile tissue paper before being sown directly into pots containing autoclave-sterilized soil. This method allowed for the direct introduction of *Agrobacterium* into the seed embryo, aiming for efficient transformation.

In the *Agro*-injection experiment, a total of 360 seeds were treated and planted in pots, with 120 seeds allocated to each genotype: PDKV Amba, Suvarna Vasundhara, and PDKV Yellow Gold. For each genotype, 60 seeds were designated for targeting the BADH2 gene and 60 for the KTI gene. The seeds were sown in sets of five per pot, with three replicates for each of the four treatment groups. In Treatment-1 (T1), injections were performed without *Agrobacterium*, serving as the control. In Treatment-2

(T2), the punchers were made at the epicotyl portion of the embryo. In Treatment-3 (T3), injections were performed at two sites: the epicotyl portion and the neck between the epicotyl and hypocotyl of the embryo. In Treatment-4 (T4), *Agro*-injection was administered at three different sites (Plate 4.2).

4.3.1 Effect of *Agro*-injection at embryo on seed germination

The germination rates of three soybean genotypes (PDKV Amba, Suvarna Vasundhara, and PDKV Yellow Gold) were evaluated under four *Agro*-injection treatments: T1 (Control), T2 (SITE-I), T3 (SITE-I + SITE-II), and T4 (SITE-I + SITE-II + SITE-III)(Table 4.3). The control (T1) resulted in the highest germination rate, with PDKV Yellow Gold showing 92.75%. The SITE-I treatment (T2) caused a slight reduction in germination for PDKV Amba and PDKV Yellow Gold, but improved germination for Suvarna Vasundhara (86.11%). Germination rates declined further in T3 and T4, especially for Suvarna Vasundhara, which had a notable drop to 43.74% in T4. These findings suggest that prolonged *Agro*-injection treatments negatively impact germination, with T1 and T2 proving to be the most effective[Table 4.3; Fig 4.3].

Table 4.3. Effect of *Argo*-injection at embryo on seed germination in experimental soybean genotypes

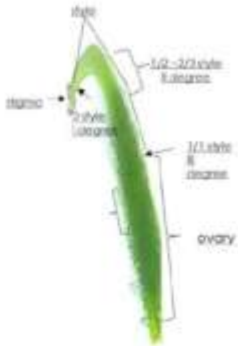
Treatments	Genotypes		
	PDKV Amba	**Suvarna Vasundhara**	**PDKV Yellow gold**
T1(Control)	92.11	83.22	92.75
T2(SITE-I)	88.78	86.11	85.45
T3(SITEI+SITE-II)	76.27	60	75.27
T4(SITE I+SITEII+SITE-III	58.59	43.74	47.52
CV			1.79
Source	SE(M))±	CD5%	
Factor A	1.32	3.87	
Factor B	1.15	3.35	
Factor AB	2.3	6.71	

A) SITE-I: At epicotyl portion of embryo

B) SITE-I + SITE-II: At epicotyl portion+ Neck of epicotyl and hypocotyl portion

C) SITE-I + SITE-II + SITE-II I : site-at epicotyl portion + neck of epicotyl and hypocotyl + hypocotyl of embryo

D) Agro-injection at floret



E) Targeting ovary

F) Pod development from infected flower

Plate 4.3:Target floral Sites for *Agro*-injection in Soybean

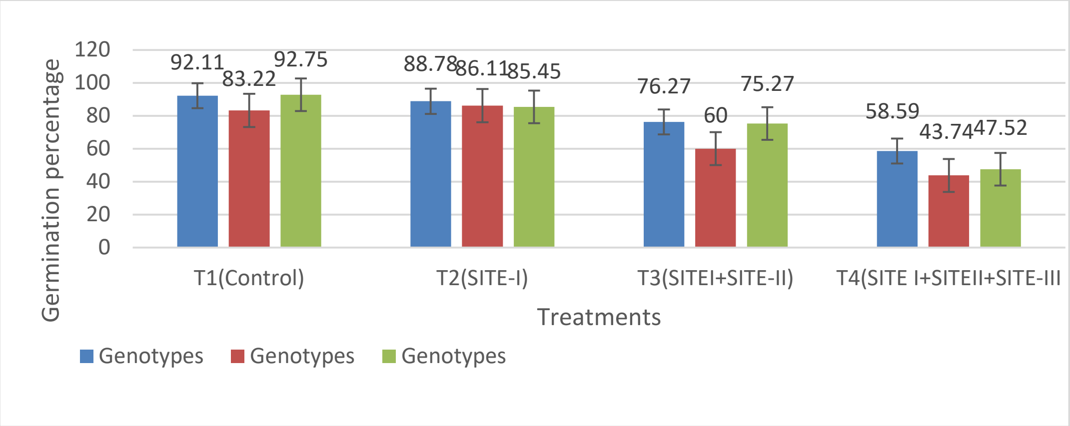


Fig 4.3. Effect of *Agro*-injection embryo treatments on seed germination in experimental soybean genotypes

4.4 Assessment of Parameters Influencing Pod Bearing Percentage in *Agro*-Injection at Floret Stage

The pod-bearing percentage, which reflects the proportion of flowers that successfully develop into pods, is a crucial indicator for evaluating the effectiveness of transformation techniques in soybean genotypes. In this study, pod-bearing percentages varied significantly across treatments and genotypes, demonstrating differential responses to the transformation protocols. For the PDKV Amba genotype, Full Seed Sonication resulted in the highest pod-bearing percentage (71.66%), suggesting a strong response to this treatment. In contrast, Half Seed Embryo Infection produced a much lower pod-bearing percentage (15.43%) for the same genotype, indicating that this technique may be less effective or that PDKV Amba has a lower receptivity to partial embryo exposure.

In Swarna Vasundhara, the Half Seed Embryo Infection technique resulted in a relatively higher pod-bearing percentage (26.92%) compared to other treatments, despite the overall low transformation efficiency in this genotype. This suggests a genotype-specific response that may Favor partial embryo exposure, though with limited pod and seed yield. For PDKV Yellow Gold, the Half Seed Embryo Infection treatment also achieved the highest pod-bearing percentage (37.62%) among the treatments, indicating that this technique may be more suitable for genotypes with greater receptivity to partial embryo infection. On the other hand, the *Agro*-injection method at exposed embryos showed inconsistent results across genotypes, with a notably high pod-bearing percentage (52.94%) in PDKV Amba, but lower efficiency in Swarna Vasundhara (9.97%) and PDKV Yellow Gold (44%). These variations in pod-bearing percentages highlight that the success of transformation protocols is highly genotype-dependent, with certain treatments promoting pod formation in specific genetic backgrounds. This genotype-specific response emphasizes the need to optimize transformation protocols for individual soybean genotypes to maximize pod development and seed yield [Table 4.4; Fig 4.4].

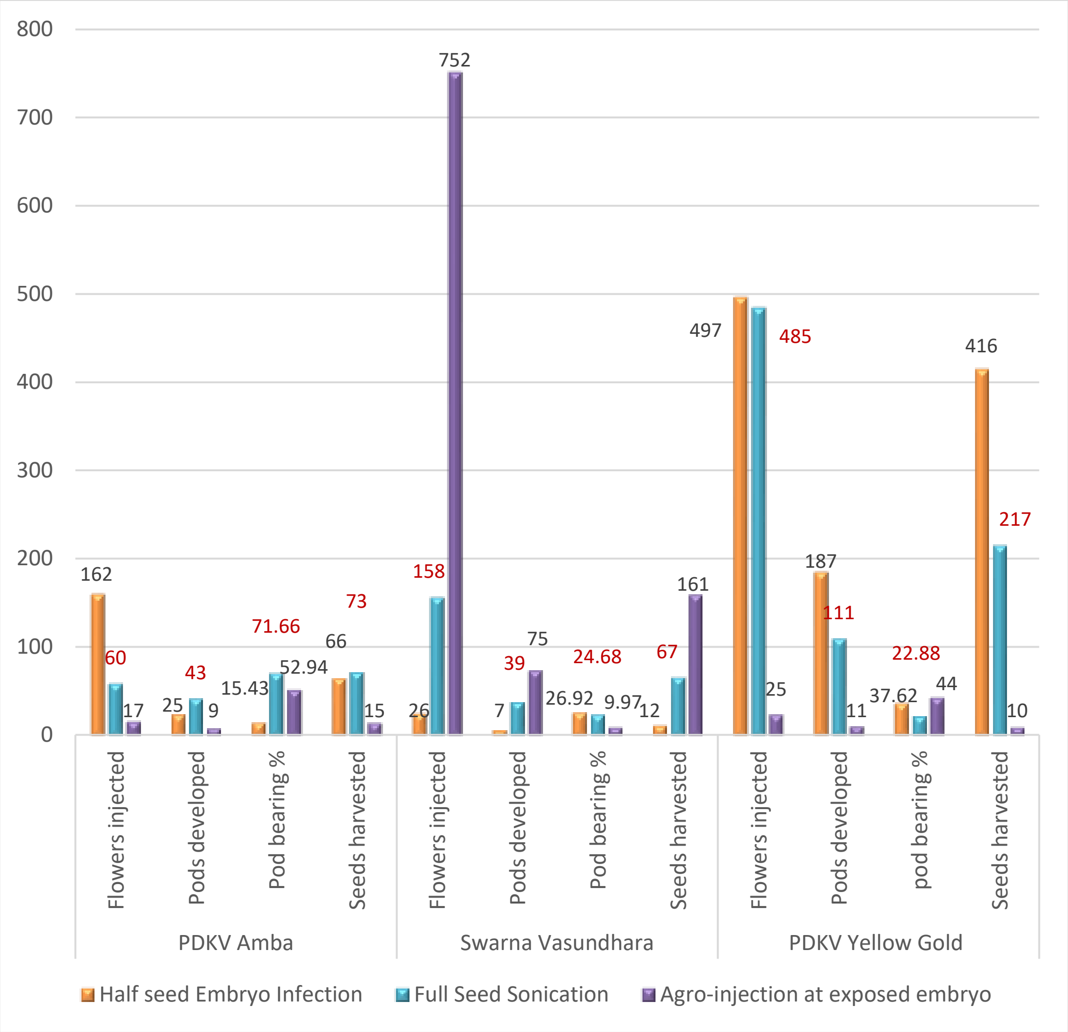


Fig 4.4. Pod bearing percentage in response to *Agro*-injection at floret across different treatment

Table 4.4: Percentage of Pod formation from different Agro-injection treatments at Floret Stage

TREATMENTS	**PDKV Amba**				**Swarna Vasundhara**				**PDKV Yellow Gold**			
	Flowers injected	**Pods developed**	**Pod bearing %**	**Seeds harvested**	**Flowers injected**	**Pods developed**	**Pod bearing %**	**Seeds harvested**	**Flowers injected**	**Pods developed**	**pod bearing %**	**Seeds harvested**
Half seed Embryo Infection	162	25	15.43	66	26	7	**26.92**	12	497	187	37.62	416
Full Seed Sonication	60	43	**71.66**	73	158	39	24.68	67	485	111	22.88	217
***Agro*-injection at exposed embryo**	17	9	52.94	15	752	75	9.97	161	25	11	**44**	10
Total no. of seeds harvested				**154**				**240**				**643**

4.5 Effect of sonication time on micro-wounds formation in explant

To evaluate the effect of sonication on the surface of explants, Scanning Electron Microscopy (SEM) was performed on explants subjected to sonication with *Agrobacterium* (Plate 4.3). Half-seed cotyledonary node explants were treated with sonication for 15 seconds, 30 seconds, 1 minute, and 2 minutes in a bath sonicator, while soybean cotyledonary node explants without sonication treatment were used as controls. The bath sonicator used in the experiment was equipped with an electronic timer. After Sonication-Assisted *Agrobacterium* Transformation (SAAT), the cotyledonary node explants were removed from the flask, placed on sterile tissue paper, and prepared for SEM analysis.

The SEM results revealed that sonication treatment induced the formation of micro-wounding on the surface of the cotyledonary node explants. Explants treated with *Agrobacterium* without sonication showed no micro-wounding. The smallest micro-wounds were observed in explants subjected to 30 seconds of sonication, compared to those treated with 15 seconds, 1 minute, and 2 minutes of sonication (Fig. 4.5). As sonication time increased, the size of the micro-wounds also increased. The largest micro-wounds were observed in explants treated with 2 minutes of sonication (Fig. 4.5).

According to Santarem et al. (1998), sonication plays a critical role in *Agrobacterium* transformation of soybean, as it may lead to browning, callus formation, or seed damage, emphasizing the need for careful control of sonication parameters to ensure stable transformation. Based on these considerations, a 30-second sonication treatment at 50 Hz frequency was selected for further experimentation in the SAAT protocol.

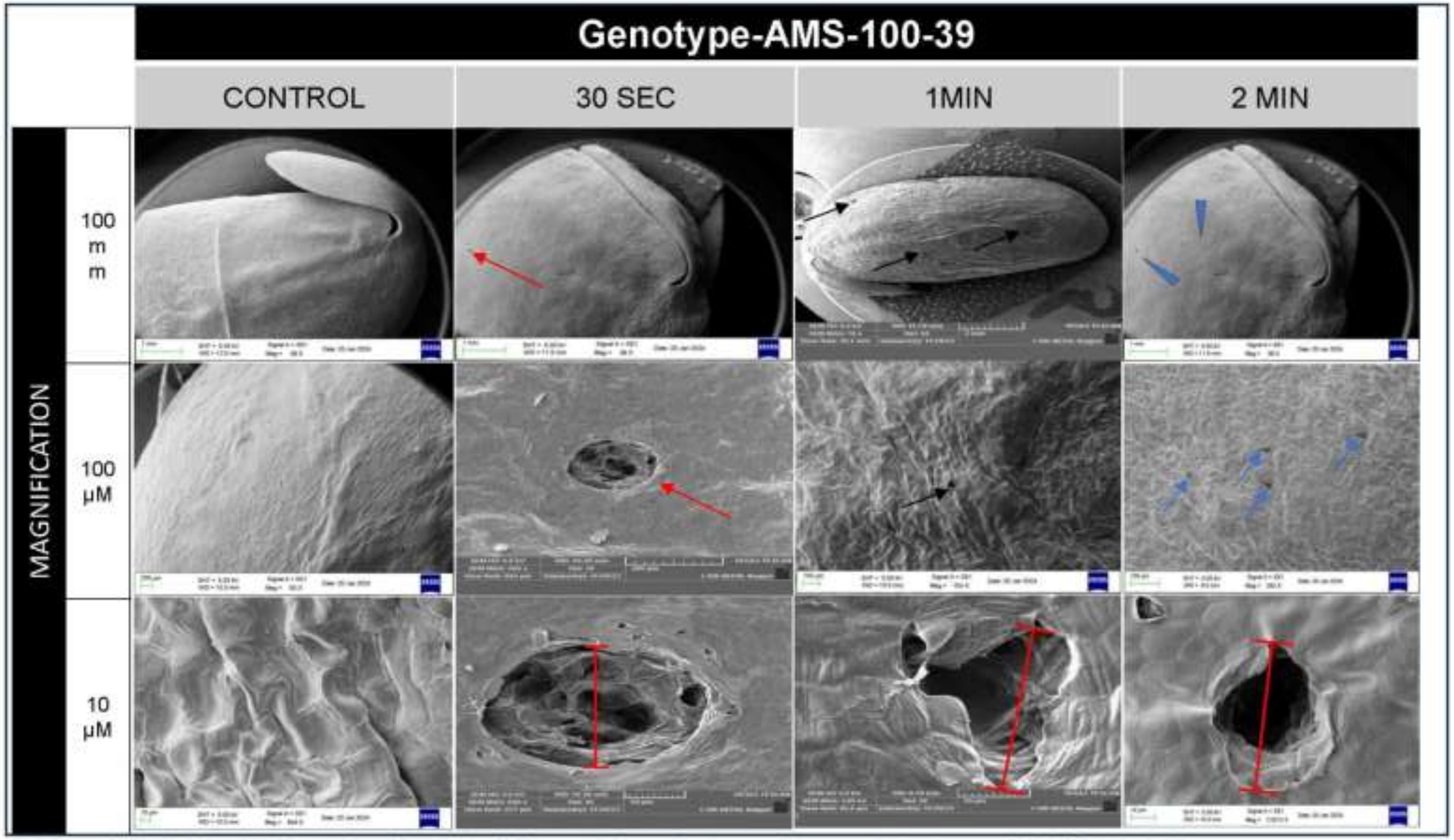


Plate 4.2. :Effect of sonication at different time interval on cotyledon of AMS-100-39

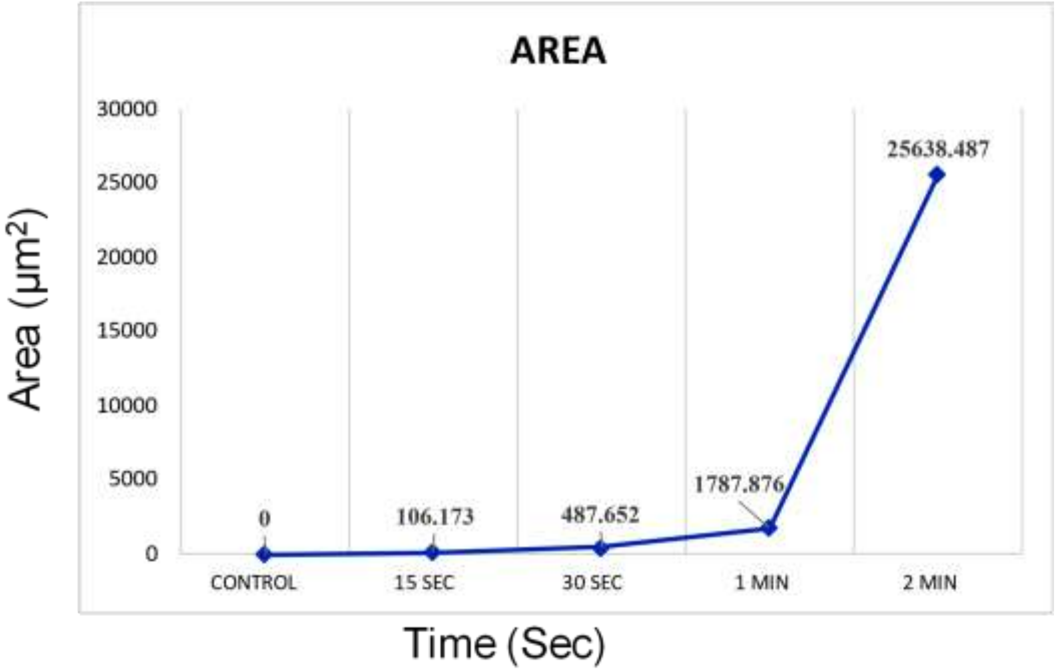


Fig 4.5. Graphical representation of the effect of sonication on explants

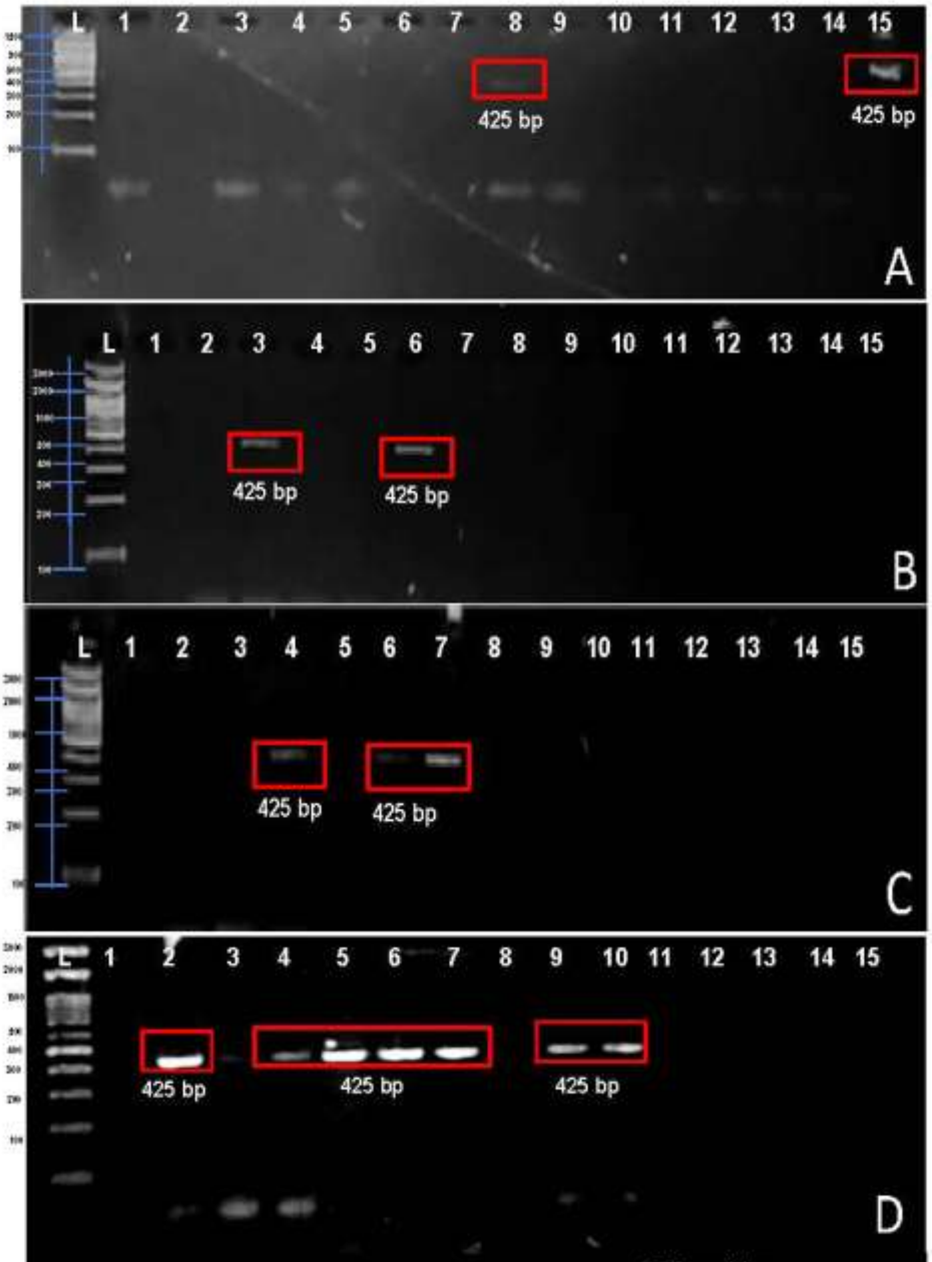
4.6 Molecular screening of putative transformants

4.6.1 PCR using *Agrobacterium* colony as template for positive control

Single colony of both *Agrobacterium* strains with respective sgRNA targeting BADH2/KTI gen was used as a template for positive control. In case of sgRNA specific primer, a product of approximately of 425 bp was found and in Cas9 Specific primer band size of 810 bp was found by performing the colony PCR that was detected upon agarose gel electrophoresis of colony PCR product.

4.7 PCR screening of putative transformants

All germinated plants were subjected to molecular screening to identify putative transformants. Genomic DNA was extracted using the Cetyl trimethyl ammonium bromide (CTAB) DNA extraction method (Rogers and Benedich, 1989) with slight modifications, and this DNA was used as a template for PCR with sgRNA-specific and Cas9-specific primers. All putative transformants yielded positive results, showing a 425 bp product for the sgRNA-specific primer and an 810 bp product for the



Gel image	Primer	Band Size	Positive control	Negative control	PCR positive	
					Code	Genotype
A	Cas9 Specific primer	425 bp	15	13,14	8	PDKV Yellow gold
B			-	14,15	3,6	PDKV Amba
C			-	14,15	4,7	Swarna Vasudhara
D			2	14,15	4,5,6,7, 9,10	PDKV Yellow gold

Plate 4.4. Screening for putative transformants using sgRNA-specific primer

Cas9-specific primer. The same bands were observed in the positive control, where transformed *Agrobacterium tumefaciens* strain EAH 105 single colony was used as a template.

4.7.1 PCR screening of putative transformants after full-seed sonication treatments

In the PDKV Amba genotype, one PCR-positive plant was identified using the Cas9-specific primer derived from 15-second sonication treatment, and one PCR-positive plant was found using the sgRNA-specific primer after a 45-second sonication treatment. For the PDKV Yellow Gold genotype, the highest number of PCR-positive plants was obtained, including one PCR-positive plant for the BADH2-targeting sgRNA-specific primer after a 30-second sonication treatment, and two PCR-positive plants for the Cas9-specific primer after 30-second and 45-second sonication treatments, respectively. No PCR-positive plants were obtained from the Swarna Vasudhara genotype [Table 4.5].

4.7.2 PCR screening of putative transformants derived from half-seed sonication treatments

A total of five PCR-positive plants were obtained from the half-seed sonication treatment. In the PDKV Amba genotype, one PCR-positive plant was identified using the sgRNA-specific primer targeting BADH2, and one PCR-positive plant was identified for the Cas9-specific primer after 15 seconds and 45 seconds of sonication. No PCR-positive plants were observed for the Swarna Vasudhara genotype.

For the PDKV Yellow Gold genotype, three PCR-positive plants were recovered: one from the 30-second sonication treatment using the sgRNA-specific primer and two from the Cas9-specific primer[Table 4.6].

4.7.3 PCR analysis of putative transformants derived from *Agro*-Injection treatments at different embryo sites

Only one PCR-positive plant was obtained from the *Agro*-injection treatment at three sites using the sgRNA-specific primer in the PDKV Amba genotype. No putative transformants were recovered from the Swarna Vasudhara and PDKV Yellow Gold genotypes[Table 4.7].

4.7.4 PCR analysis of putative transformants derived from *Agro*-injection treatment at floret

Highest PCR positive putative transformants were recorded for *Agro*-injection treatment with total 15 PCR positive plants in which 3 PCR positive for BADH2 targeting sgRNA specific primer and 1 for Cas9 specific primer for PDKV Ambs genotype. For Swarna Vasudhara we got 1 PCR positive for sgRNA specific and 3 Cas9 specific primer positive plant and in PDKV Yellow gold genotype 7 PCR positive out of which 4 for sgRNA and 3 for Cas9 specific primer[Table 4.8].

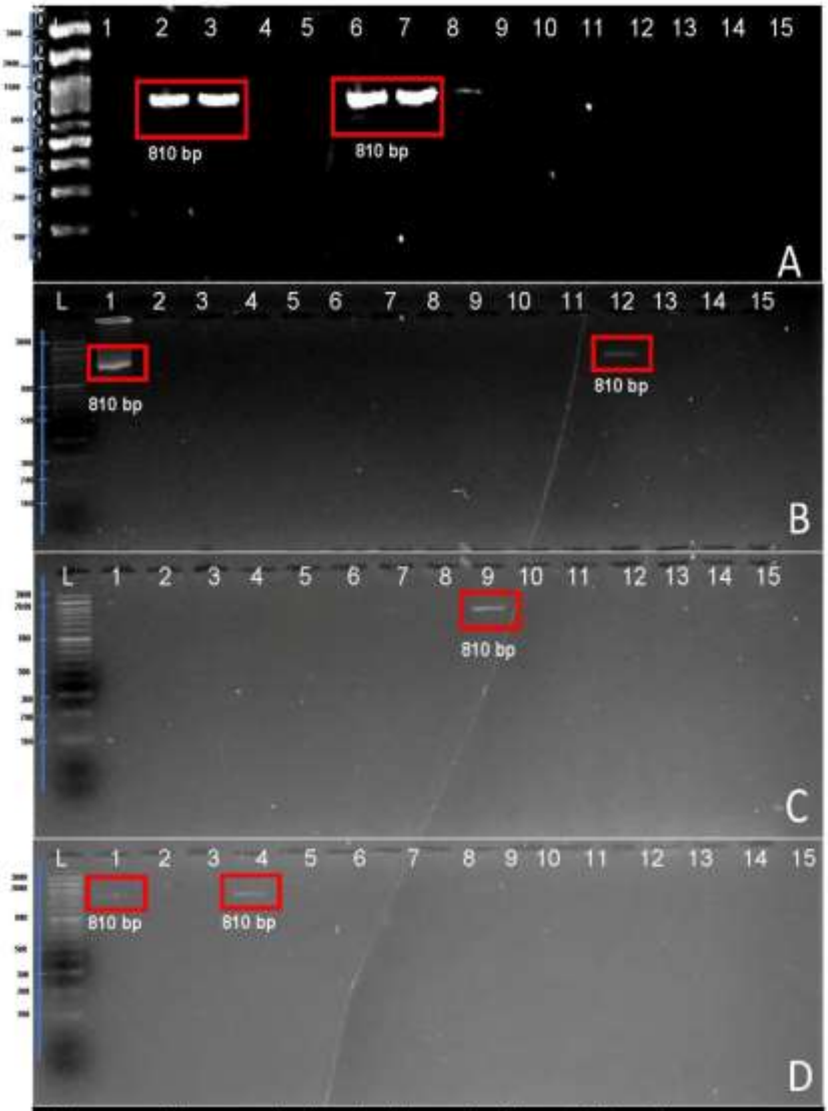
4.8 Assessment of CRISPR/Cas9 delivery efficiency across all treatments

In this study, each PCR-positive plant was considered a single transformation event to calculate transformation efficiency. For the full-seed sonication treatment, six PCR-positive plants were obtained from 120 attempts in the PDKV Amba genotype, resulting in a transformation efficiency of 1.7%. In the PDKV Yellow Gold genotype, four PCR-positive plants were recorded out of 120 attempts, yielding a transformation efficiency of 3.3% across all sonication treatments. In half seed sonication treatment transformation percentage of 2.5% was obtain in PDKV yellow gold and 1.7% transformation percentage in PDKV Amba.

For *Agro*-injection at the embryo treatment, only one PCR-positive plant was recovered, resulting in a transformation efficiency of 0.8% in the PDKV Amba genotype.

For *Agro*-injection at the floret treatment, 154 seeds were sown, and four PCR-positive plants were recovered, yielding a transformation efficiency of 2.6% in the Swarna Vasudhara genotype. In the PDKV Yellow Gold genotype, 643 seeds were sown, and seven PCR-positive plants were obtained. This resulted in a transformation efficiency of 1.1%[Table 4.9,Fig 4.6].

Molecular analysis employing polymerase chain reaction clearly indicate the integration of transgenes from the T-DNA region of



Gel image	Primer	Band Size	Positive control	Negative control	PCR positive	
					Code	Genotype
A	Cas9 Specific primer	810 bp	2	14,15	3,6,7	PDKV Yellow gold
B			1	14,15	12	PDKV Amba
C			-	14,15	9	Swarna Vasudhara
D			1	14,15	4	PDKV Yellow gold

Plate 4.5. Screening for putative transformants using Cas9-specific primer

Agrobacterium to the soybean host genome employing the investigated *in-planta* transformation methods.

Similar findings were reported by Liu et al. (2009), who achieved a transformation efficiency of 3.2% using ovary tissue as the target. Likewise, Zia et al. employed *Agro*-injection on two soybean cultivars, NARC-7 and NARC-4, achieving transformation efficiencies of 14.2% and 6.45%, respectively. These findings provide a strong basis to suggest that Sonication-Assisted *Agrobacterium*-Mediated delivery of the CRISPR/Cas9 construct may offer a slight advantage over *Agro*-injection. However, both methods demonstrate successful potential for genetic transformation and can be effectively utilized for this purpose.

Table 4.5. PCR screening of plants derived from full-seed sonication Treatments

GENOTYPES	PDKV Amba				Swarna Vasudhara				PDKV Yellow gold			
	No. of attempts	PCR positive			No. of attempts	PCR positive			No. of attempts	PCR positive		
TREATMENT		GmBADH gRNA	KTI gRNA	Cas9		GmBADH gRNA	KTI gRNA	Cas9		GmBADH gRNA	KTI gRNA	Cas9
T1(Control)	30	0	0	0	30	0	0	0	30	0	0	0
T2(15 sec)	30	0	0	0	30	0	0	0	30	0	0	0
T3(30 sec)	30	1	0	1	30	0	0	0	30	0	0	3
T4(45 sec	30	0	0	0	30	0	0	0	30	1	0	0
TOTAL	120	1	0	1	120	0	0	0	120	1	0	3

Table 4.6. PCR positive plants derived from half-seed sonication treatment

GENOTYPES	PDKV Amba				Swarna Vasudhara				PDKV Yellow gold			
TREATMENT	No. of attempts	PCR positive			No. of attempt	PCR positive			No. of attempt	PCR positive		
		GmBADH gRNA	KTI gRNA	Cas9		GmBADH gRNA	KTI gRNA	Cas9		GmBADH gRNA	KTI gRNA	Cas9
T1(Control)	30	0	0	0	30	0	0	0	30	0	0	0
T2(15 sec)	30	0	0	1	30	0	0	0	30	0	0	0
T3(30 sec)	30	0	0	0	30	0	0	0	30	1	0	1
T4(45 sec	30	1	0	0	30	0	0	0	30	0	0	1
TOTAL	120	1	0	1	120	0	0	0	120	1	0	2

Table 4.7. PCR positive for every treatment of *Agro*-injection at Embryo

GENOTYPES	PDKV Amba				Swarna Vasudhara				PDKV Yellow gold			
		PCR positive				PCR positive				PCR positive		
TREATMENT	No. of attempts	GmBADH gRNA	KTI gRNA	Cas9	No. of attempts	GmBADH gRNA	KTI gRNA	Cas9	No. of attempts	GmBADH gRNA	KTI gRNA	Cas9
T1(Control)	30	0	0	0	30	0	0	0	30	0	0	0
T2(SITE-I)	30	0	0	0	30	0	0	0	30	0	0	0
T3(SITEI+SITE-II)	30	0	0	0	30	0	0	0	30	0	0	0
T4 (SITE I+SITEII+SITE-III	30	1	0	0	30	0	0	0	30	0	0	0
TOTAL	120	1	0	0	120	0	0	0	120	0	0	0

Table 4.8. PCR positives plants derived from *Agro*-Injection treatments at Floret

GENOTYPES	PDKV Amba				Swarna Vasudhara				PDKV Yellow gold			
TREATMENT	**No. of attempts**	**PCR positive**			**No. of attempts**	**PCR positive**			**No. of attempts**	**PCR positive**		
		GmBADH gRNA	**KTI gRNA**	**Cas9**		**GmBADH gRNA**	**KTI gRNA**	**Cas9**		**GmBADH gRNA**	**KTI gRNA**	**Cas9**
FULL SEED SONICATION	66	2	0	1	12	0	0	0	416	3	0	2
HALF SEED SONICATION	73	1	0	0	67	0	0	1	217	1	0	1
AGRO-INJECTION	15	0	0	0	161	1	0	2	10	0	0	0
TOTAL	154	3	0	1	240	1	0	3	643	4	0	3

Table 4.9. Response of experimental soybean genotypes to *In-Planta* transformation

Treatment	PDKV Amba			Swarna Vasudhara			PDKV Yellow gold		
	No. of seed sown	**No. PCR positive plant**	**CRISPR\Cas9 delivery percentage**	**No. of seed sown**	**No. PCR positive plant**	**CRISPR\Cas9 delivery percentage**	**No. of seed sown**	**No. PCR positive plant**	**CRISPR\Cas9 delivery percentage**
Full seed sonication	120	2	1.7	120	0	0	120	4	3.3
Half seed sonication	120	2	1.7	120	0	0	120	3	2.5
***Agro*-injection at exposed embryo**	120	1	0.8	120	0	0	120	0	0
***Agro*-injection at floret**	154	4	2.6	240	4	1.7	643	7	1.1

Fig 4.6. Response of experimental soybean genotypes to *In-Planta* transformation

CHAPTER V

SUMMARY AND CONCLUSION

The study titled "Assessment of Critical Parameters Associated with *In-Planta* Transformation of Elite Soybean Genotypes (*Glycine max* L.)" aimed to evaluate the effectiveness of two *in-planta* transformation techniques: Sonication-Assisted *Agrobacterium*-Mediated Transformation and *Agro*-Injection. The investigation focused on three soybean genotypes, including one vegetable-type variety, Swarna Vasundhara (SV), and two grain-type varieties, Swarna Soya (AMS MB 5-18) and PDKV Yellow Gold (AMS 1001). The primary objectives were to identify the superior transformation method and analyze key parameters influencing the success of *in-planta* transformation in these genotypes.

The study revealed that the germination percentage of soybean seeds is significantly affected by both the duration of sonication treatment and the genotype. For full-seed transformations, the genotype PDKV Yellow Gold demonstrated the highest germination rates, achieving 95.15% in the control group and 93.45% with a 15-second sonication treatment. However, prolonged sonication led to a progressive decline in germination, with the lowest rate of 41.33% recorded for PDKV Yellow Gold at 45 seconds.

Similarly, in half-seed sonication treatments, PDKV Yellow Gold outperformed other genotypes, achieving a germination rate of 90.85% in the control group. Although a slight decrease was observed with 15-second sonication, it remained the most efficient genotype across all treatments. These findings highlight the critical role of optimizing sonication duration and selecting appropriate genotypes to enhance germination outcomes in *in-planta* transformation experiments. Prolonged sonication exposure (30 and 45 seconds) resulted in a significant reduction in germination rates, with Swarna Vasundhara showing a decline to 57.7% and 37.7% under 30- and 45-second treatments, respectively. This indicates that extended sonication negatively impacts germination regardless of genotype, highlighting the importance of shorter sonication durations to preserve seed viability. For *Agro*-injection treatments, targeting specific embryo sites significantly influenced germination rates. The highest germination (92.27%) was recorded in PDKV Yellow Gold for the T-2 treatment (single embryo site), followed by PDKV Amba in the control group (92.11%). These findings underscore the importance of precise targeting and shorter treatment durations for optimal germination outcomes.

Agro-injection at the floret stage targeting the ovary was evaluated for its effect on pod-bearing percentage across treatments. The highest pod-bearing percentage was observed in PDKV Amba (71.66%) under full-seed sonication treatment, followed by 52.94% for the same genotype under *Agro*-injection targeting the exposed embryo. These variations in pod-bearing percentages underscore the genotype-specific nature of transformation success, highlighting the importance of tailoring protocols to individual soybean genotypes to enhance pod formation and seed yield.

Scanning Electron Microscopy (SEM) analysis of sonicated explants revealed that sonication induces micro-wounding on the surface of cotyledonary node explants, with the extent of damage increasing with longer sonication intervals. Minimal micro-wounding was observed with the 30-second treatment, whereas the 2-minute treatment resulted in extensive surface damage. These findings emphasize the importance of optimizing sonication duration to balance effective transformation with minimal tissue damage.

Molecular screening of putative transformants through PCR analysis confirmed successful T-DNA integration into the soybean genome, with transformation efficiency varying across techniques and genotypes. The highest efficiency (3.3%) was observed in PDKV Yellow Gold using the full-seed sonication method, highlighting the genotype's responsiveness to this approach. *Agro*-injection at the floret stage achieved transformation rates of 2.6% in PDKV Yellow Gold and 1.7% in Swarna Vasundhara, while half-seed sonication resulted in 2.5% efficiency in PDKV Yellow Gold and 1.7% in PDKV Amba. The lowest efficiencies were recorded with *Agro*-injection targeting multiple sites, underscoring the need to refine protocols for higher success rates.

CHAPTER VI

IMPLICATIONS

The sonication-assisted *Agrobacterium*-mediated method shows promise for delivering CRISPR/Cas9 in soybean genome editing, while the *Agro*-injection method requires refinement to improve efficiency and consistency across genotypes.

Validation of genome editing can be enhanced through sequencing and biochemical analysis of PCR-positive mutants. As a cost-effective alternative to labor-intensive tissue culture methods, *in-planta* transformation offers a practical approach to increase transformants and boost transformation efficiency in recalcitrant crops like soybean.

CHAPTER VII

LITERATURE CITED

Arikit, S., Yoshihashi, T., Wanchana, S., Uyen, T. T., Huong, N. T., Wongpornchai, S. and Vanavichit, A. 2011. A deficiency in the amino aldehyde dehydrogenase encoded by GmAMADH2, the homologue of rice *Os*2AP, enhances 2-acetyl-1-pyrroline biosynthesis in soybeans (*Glycine max L*.). *Plant Biotechnol J*. 9(1): 75-87.

Arun, M., Subramanyam, K., Mariashibu, T.S., Theboral, J., Shivanandhan, G., Manickavasagam, M. and Ganapathi, A., 2015. Application of sonication in combination with vacuum infiltration enhances the *Agrobacterium*-mediated genetic transformation in Indian soybean cultivars. Applied biochemistry and biotechnology, 175, pp.2266-2287.

Bechtold, N., 1993. *In planta Agrobacterium*-mediated gene transfer by infiltration of adult Arabidopsis thaliana plants. CR Acad. Sci. Paris, Life Sci., 316, pp.1194-1199.

Bent, A.F., 2000. Arabidopsis in planta transformation. Uses, mechanisms, and prospects for transformation of other species. *Plant physiology*, *124*(4), pp.1540-1547.

Bradbury, L. M., Fitzgerald, T. L., Henry, R. J., Jin, Q. & Waters, D. L. 2005. The gene for fragrance in rice. *Plant Biotechnol J*. 3:363-370

Broothaerts, W., Mitchell, H.J., Weir, B., Kaines, S., Smith, L.M., Yang, W., Mayer, J.E., Roa-Rodriguez, C. and Jefferson, R.A., 2005. Gene transfer to plants by diverse species of bacteria. *Nature*, *433*(7026), pp.629-633.

Cai, Z., Xian, P., Cheng, Y., Yang, Y., Zhang, Y., He, Z., Xiong, C., Guo, Z., Chen, Z., Jiang, H. and Ma, Q., 2023. Natural variation of GmFATA1B regulates seed oil content and composition in soybean. *Journal of Integrative Plant Biology*, *65*(10), pp.2368-2379.

Chee, P.P., Fober, K.A. and Slightom, J.L., 1989. Transformation of soybean (Glycine max) by infecting germinating seeds with Agrobacterium tumefaciens. *Plant physiology*, *91*(3), pp.1212-1218.

Chen, G., Zeng, F., Wang, J., Ye, X., Zhu, S., Yuan, L., Hou, J. and Wang, C., 2019. Transgenic Wucai (Brassica campestris L.) produced via Agrobacterium-mediated anther transformation in planta. *Plant cell reports*, *38*, pp.577-586.

Chen, L., Cai, Y., Liu, X., Yao, W., Guo, C., Sun, S., Wu, C., Jiang, B., Han, T. and Hou, W., 2018. Improvement of soybean *Agrobacterium*-mediated transformation efficiency by adding glutamine and asparagine into the culture media. International Journal of Molecular Sciences, 19(10), p.3039.

Chen, M., Shen, S., Li, Z., Wang, H., Wang, J., Yang, G., Yang, W., Deng, L., Gong, D. and Zhang, J., 2024. CRISPR/Cas9-Mediated Targeted

Mutagenesis of Betaine Aldehyde Dehydrogenase 2 (BADH2) in Tobacco Affects 2-Acetyl-1-pyrroline. *Agronomy*, *14*(2), p.321.

Cho, Hyeon-Je, York Moy, Nathan A. Rudnick, Theodore M. Klein, Jiaming Yin, Joy Bolar, Carol Hendrick et al. "Development of an efficient marker-free soybean transformation method using the novel bacterium Ochrobactrum haywardense H1." *Plant Biotechnology Journal* 20, no. 5 (2022): 977-990.

Choi, S.W., Ly, S., Lee, J.H., Oh, H.S., Kim, S.Y., Kim, N.H. and Chung, J.I., 2022. Breeding of penta null soybean [Glycine max (L.) Merr.] for five antinutritional and allergenic components of lipoxygenase, kTI, lectin, 7S α′ subunit, and stachyose. *Frontiers in Plant Science*, *13*, p.910249.

Christou, P., McCabe, D.E. and Swain, W.F., 1988. Stable transformation of soybean callus by DNA-coated gold particles. *Plant Physiology*, *87*(3), pp.671-674.

Curtis, I.S. and Nam, H.G., 2001. Transgenic radish (Raphanus sativus L. longipinnatus Bailey) by floral-dip method–plant development and surfactant are important in optimizing transformation efficiency. *Transgenic research*, *10*, pp.363-371.

Das, P. and Joshi, N.C., 2011. Minor modifications in obtainable Arabidopsis floral dip method enhances transformation efficiency and production of homozygous transgenic lines harboring a single copy of transgene. *Advances in Bioscience and Biotechnology*, *2*(02), p.59.

FAO (Food and Agriculture Organization), 2003. http://apps.fao.org.

Fitzgerald, T. L., Waters, D. L. & Henry, R. J. 2009.Betaine aldehyde dehydrogenase in plants. *Plant biology* . 11: 119–130

Hada, A., Krishnan, V., Mohamed Jaabir, M.S., Kumari, A., Jolly, M., Praveen, S. and Sachdev, A., 2018. Improved *Agrobacterium* tumefaciens-mediated transformation of soybean [Glycine max (L.) Merr.] following optimization of culture conditions and mechanical techniques. *In Vitro Cellular & Developmental Biology-Plant*, *54*, pp.672-688.

Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C. and Cottage, A., 2006. A rapid and robust method of identifying transformed Arabidopsis thaliana seedlings following floral dip transformation. *Plant methods*, *2*, pp.1-7.

Hattori H, Ashida A, Itô C, Yoshida M. Determination of molybdenum in foods and human milk, and an estimate of average molybdenum intake in the Japanese population. *J Nutr Sci Vitaminol (Tokyo)*. 2004;50(6):404-409. doi:10.3177/jnsv.50.404

Hinchee, M., Connor-Ward, D., Newell, C. *et al.* Production of Transgenic Soybean Plants Using *Agrobacterium*-Mediated DNA Transfer. *Nat Biotechnol* **6**, 915–922 (1988). https://doi.org/10.1038/nbt0888-915

Hirsch, A.M., Lee, A., Deng, W. and Tucker, S.C., 2010. An open-flower mutant of Melilotus alba: Potential for floral-dip transformation of a papilionoid legume with a short life cycle?. *American journal of botany*, *97*(3), pp.395-404.

Holsters M, De Waele D, Depicker A, Messens E, Van Montagu M and Schell J (1978) Transfection and transformation of *Agrobacterium tumefaciens*. Molecular and General Genetics MGG, 163(2), 181-187.

Hu, C. Y., and Wang, L. (1999). *In planta* soybean transformation technologiesdeveloped in China: procedure, confirmation and field performance. In Vitro

Joersbo, M. and Brunstedt, J., 1990. Direct gene transfer to plant protoplasts by mild sonication. *Plant cell reports*, *9*, pp.207-210.

Jofuku, K.D., Schipper, R.D. and Goldberg, R.B., 1989. A frameshift mutation prevents Kunitz trypsin inhibitor mRNA accumulation in soybean embryos. The Plant Cell, 1(4), pp.427-435.

Juwattanasomran, R., Somta, P., Chankaew, S., Shimizu, T., Wongpornchai, S., Kaga, A. and Peerasak, S. 2010. A SNP in *Gm*BADH2 gene associates with fragrance in vegetable soybean variety ‘Kaori’ and SNAP marker development for the fragrance. *Theor Appl Genet*. 122: 533-541.

Juwattanasomran, R., Somta, P., Chankaew, S., Shimizu, T., Wongpornchai, S., Kaga, A. and Peerasak, S. 2010. Identification of a new fragrance allele in soybean and development of its functional marker. *Mol Breeding*. 29:13-21.

Juwattanasomran, R., Somta, P., Chankaew, S., Shimizu, T., Wongpornchai, S., Kaga, A. and Srinives, P., 2011. A SNP in GmBADH2 gene associates with fragrance in vegetable soybean variety “Kaori” and SNAP marker development for the fragrance. *Theoretical and applied genetics*, *122*, pp.533-541.

Juwattanasomran, R., Somta, P., Chankaew, S., Shimizu, T., Wongpornchai, S., Kaga, A. and Peerasak, S. 2010. A SNP in *Gm*BADH2 gene associates with fragrance in vegetable soybean variety ‘Kaori’ and SNAP marker development for the fragrance. *Theor Appl Genet*. 122: 533-541.

Juwattanasomran, R., Somta, P., Kaga, A. et al. Identification of a new fragrance allele in soybean and development of its functional marker. Mol Breeding 29, 13–21 (2012). https://doi.org/10.1007/s11032-010-9523-0

Kalbande, B.B. and Patil, A.S., 2016. Plant tissue culture independent Agrobacterium tumefaciens mediated In-planta transformation strategy for upland cotton (Gossypium hirsutum). *Journal of Genetic Engineering and Biotechnology*, *14*(1), pp.9-18.

Karthik, S., Pavan, G. and Manickavasagam, M., 2020. Nitric oxide donor regulates *Agrobacterium*-mediated genetic transformation efficiency in soybean [Glycine max (L.) Merrill]. Plant Cell, Tissue and Organ Culture (PCTOC), 141, pp.655-660.

Kesiraju, K., Tyagi, S., Mukherjee, S., Rai, R., Singh, N.K., Sreevathsa, R. and Dash, P.K., 2021. An apical meristem-targeted in planta transformation method for the development of transgenics in flax

(Linum usitatissimum): optimization and validation. *Frontiers in Plant Science*, *11*, p.562056.

Kim, W.S., Gillman, J.D., Kim, S., Liu, J., Janga, M.R., Stupar, R.M. and Krishnan, H.B., 2024. Bowman–Birk Inhibitor Mutants of Soybean Generated by CRISPR-Cas9 Reveal Drastic Reductions in Trypsin and Chymotrypsin Inhibitor Activities. International Journal of Molecular Sciences, 25(11), p.5578.

Li, J., Todd, T.C. and Trick, H.N., 2010. Rapid in planta evaluation of root expressed transgenes in chimeric soybean plants. *Plant cell reports*, *29*, pp.113-123.

Li, S., Cong, Y., Liu, Y., Wang, T., Shuai, Q., Chen, N., Gai, J. and Li, Y., 2017. Optimization of *Agrobacterium*-mediated transformation in soybean. Frontiers in plant science, 8, p.246.

Liener IE. Implications of antinutritional components in soybean foods. Crit Rev Food Sci Nutr. 1994;34(1):31-67. doi:10.1080/10408399409527649

Liu, M., Yang, J., Cheng, Y.Q. and An, L.J., 2009. Optimization of soybean (Glycine max (L.) Merrill) in planta ovary transformation using a linear minimal gus gene cassette. *Journal of Zhejiang University Science B*, *10*, pp.870-876.

Mangena, P., 2019. A simplified in-planta genetic transformation in soybean. *Research Journal of Biotechnology Vol*, *14*, p.9.

McCabe, D.E., Swain, W.F., Martinell, B.J. and Christou, P., 1988. Stable transformation of soybean (Glycine max) by particle acceleration. *Bio/technology*, *6*(8), pp.923-926.

Mittal, P., Kumar, V., Anita, R.A.N.I. and Gokhale, S.M., 2021. Bowman-Birk inhibitor in soybean: genetic variability in relation to total trypsin inhibitor activity and elimination of Kunitz trypsin inhibitor. *Notulae Scientia Biologicae*, *13*(1), pp.10836-10836.

Nakashima, K., Shinwari, Z.K., Sakuma, Y., Seki, M., Miura, S., Shinozaki, K. and Yamaguchi-Shinozaki, K., 2000. Organization and expression of two Arabidopsis DREB2 genes encoding DRE-binding proteins involved in dehydration-and high-salinity-responsive gene expression. *Plant molecular biology*, *42*, pp.657-665.

Olhoft, P.M., Donovan, C.M. and Somers, D.A., 2006. Soybean (Glycine max) transformation using mature cotyledonary node explants. *Agrobacterium Protocols*, pp.385-396.

Pagano, M.C. and Miransari, M., 2016. The importance of soybean production worldwide. In *Abiotic and biotic stresses in soybean production* (pp. 1-26). Academic Press.

Panse, V. G. and P. V. Sukhatme. 1978.Statistical Methods for AgriculturalWorkers. ICAR, New Delhi.

Pareddy, D., Chennareddy, S., Anthony, G., Sardesai, N., Mall, T., Minnicks, T., Karpova, O., Clark, L., Griffin, D., Bishop, B. and Shumway, N.,

2020. Improved soybean transformation for efficient and high throughput transgenic production. Transgenic Research, 29, pp.267-281.

Paz, M.M., Shou, H., Guo, Z., Zhang, Z., Banerjee, A.K., and Wang, K. (2004). Assessment of conditions affecting Agrobacterium-mediated soybean transformation using the cotyledonary node explant.Euphytica 136:167–179.

Pesic, M.B., Vucelic-Radovic, B.V., Barac, M.B., Stanojevic, S.P. and Nedovic, V.A., 2007. Influence of different genotypes on trypsin inhibitor levels and activity in soybeans. Sensors, 7(1), pp.67-74.

Qian, L., Jin, H., Yang, Q., Zhu, L., Yu, X., Fu, X., Zhao, M. and Yuan, F., 2022. A sequence variation in GmBADH2 enhances soybean aroma and is a functional marker for improving soybean flavor. *International Journal of Molecular Sciences*, *23*(8), p.4116.

Qin, P., Wang, T. and Luo, Y., 2022. A review on plant-based proteins from soybean: Health benefits and soy product development. *Journal of Agriculture and Food Research*, *7*, p.100265.

Ratanasut, K., Rod-In, W. and Sujipuli, K., 2017. In planta Agrobacterium-mediated transformation of rice. *Rice Science*, *24*(3), pp.181-186.

Rogers, S.O. and Bendich, A.J., 1989. Extraction of DNA from plant tissues. *Plant molecular biology manual*, pp.73-83.

Roychaudhuri, R., Sarath, G., Zeece, M. and Markwell, J., 2004. Stability of the allergenic soybean Kunitz trypsin inhibitor. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, *1699*(1-2), pp.207-212.

Santarem, E.R., Trick, H.N., Essig, J.S. and Finer, J.J., 1998. Sonication-assisted *Agrobacterium*-mediated transformation of soybean immature cotyledons: optimization of transient expression. *Plant Cell Reports*, *17*, pp.752-759.

Shah, S.H., Ali, S., Jan, S.A. and Ali, G.M., 2015. Piercing and incubation method of in planta transformation producing stable transgenic plants by overexpressing DREB1A gene in tomato (Solanum lycopersicum Mill.). *Plant Cell, Tissue and Organ Culture (PCTOC)*, *120*, pp.1139-1157.

Shea MK, Booth SL. Update on the role of vitamin K in skeletal health. *Nutr Rev*. 2008;66(10):549-557. doi:10.1111/j.1753-4887.2008.00106.x

Shinwari, Z.K., Jan, S.A., Nakashima, K. and Yamaguchi-Shinozaki, K., 2020. Genetic engineering approaches to understanding drought tolerance in plants. *Plant Biotechnology Reports*, *14*(2), pp.151-162.

Sim, J., Kuwabara, C., Sugano, S., Adachi, K. and Yamada, T., 2023. Recent advances in the improvement of soybean seed traits by genome editing. *Plant Biotechnology*, *40*(3), pp.193-200.

Solliman, M.E.D., Mohasseb, H.A.A., Al-Khateeb, A.A., Al-Khateeb, S.A., Shehata, W.F. and Aldaej, M.I., 2021. *Agro-injection* technique for transient gene expression as rapid and highly efficient method for potato *Agrobacterium*-based transformation by Cry1Ca gene.

Sudarić, A., Vratarić, M. and Sudar, R., 2009. Genetic improvement of soybean by modern breeding strategies in region of the Eastern Croatia. In *World Soybean Research Conference VIII* (pp. 54-54).

Supartana, P., Shimizu, T., Shioiri, H., Nogawa, M., Nozue, M. and Kojima, M., 2005. Development of simple and efficient in planta transformation method for rice (Oryza sativa L.) using Agrobacterium tumefaciens. *Journal of bioscience and bioengineering*, *100*(4), pp.391-397.2006. In planta Agrobacterium-mediated transformation by vacuum infiltration. *Arabidopsis protocols*, pp.215-223.

UnitedStates Department of Agriculture,USDA FAS Report ,2024(https://fas.usda.gov/data/production/commodity/2222000)

Tian, Y., Zhou, Y., Gao, G., Zhang, Q., Li, Y., Lou, G. and He, Y., 2023. Creation of two-line fragrant glutinous hybrid rice by editing the Wx and OsBADH2 genes via the CRISPR/Cas9 system. *International journal of molecular sciences*, *24*(1), p.849.

Trick, H., Finer, J. Sonication-assisted *Agrobacterium*-mediated transformation of soybean [Glycine max (L.) Merrill] embryogenic suspension culture tissue. Plant Cell Reports 17, 482–488 (1998). https://doi.org/10.1007/s002990050429

Trieu, A.T., Burleigh, S.H., Kardailsky, I.V., Maldonado-Mendoza, I.E., Versaw, W.K., Blaylock, L.A., Shin, H., Chiou, T.J., Katagi, H., Dewbre, G.R. and Weigel, D., 2000. Transformation of Medicago truncatula via infiltration of seedlings or flowering plants with Agrobacterium. *The plant journal*, *22*(6), pp.531-541.

Wang, W.C., Menon, G. and Hansen, G., 2003. Development of a novel Agrobacterium-mediated transformation method to recover transgenic Brassica napus plants. *Plant cell reports*, *22*(4), pp.274-281.

Wang, Y., Li, Z., Chen, X. et al. An efficient soybean transformation protocol for use with elite lines. Plant Cell Tissue Organ Cult 151, 457–466 (2022). https://doi.org/10.1007/s11240-022-02312-6

Wang, Z., Shea, Z., Rosso, L., Shang, C., Li, J., Bewick, P., Li, Q., Zhao, B. and Zhang, B., 2022. Development of molecular markers of the Kunitz trypsin inhibitor mutant alleles generated by CRISPR/Cas9-mediated mutagenesis in soybean. *bioRxiv*, pp.2022-08.

Wang, Z., Shea, Z., Rosso, L., Shang, C., Li, J., Bewick, P., Li, Q., Zhao, B. and Zhang, B., 2023. Development of new mutant alleles and markers for KTI1 and KTI3 via CRISPR/Cas9-mediated mutagenesis to reduce trypsin inhibitor content and activity in soybean seeds. *Frontiers in Plant Science*, *14*, p.1111680.

Warburton M (2005) Laboratory Protocols CIMMYT Applied Molecular Genetics Laboratory (No. 56184). CIMMYT: International Maize and Wheat Improvement Center.

Weigel, D. and Glazebrook, J., 2006. Vectors and Agrobacterium hosts for Arabidopsis transformation. *Cold Spring Harbor Protocols*, *2006*(7), pp.pdb-ip29.

Wu, T., Xu, X., Zhang, L., Yuan, S., Chen, F., Sun, S. and Jiang, B., 2024. Progress and future impacts on genomic dissection of soybean domestication and improvement. *Critical Reviews in Plant Sciences*, *43*(2), pp.116-130.

Xu, Z., Ye, Z., Li, Y., Li, J. and Liu, Y., 2020. Comparative study of the oxidation stability of high oleic oils and palm oil during thermal treatment. *Journal of Oleo Science*, *69*(6), pp.573-584.

Yang, X.F., Yu, X.Q., Zhou, Z., Ma, W.J. and Tang, G.X., 2016. A high-efficiency *Agrobacterium* tumefaciens mediated transformation system using cotyledonary node as explants in soybean (Glycine max L.). Acta physiologiae plantarum, 38, pp.1-10.

Zhang, L.J., Cheng, L.M., Xu, N., Zhao, N.M., Li, C.G., Yuan, J. and Jia, S.R., 1991. Efficient transformation of tobacco by ultrasonication. *Bio/technology*, *9*(10), pp.996-997.

Zhang, W., Zuo, Z., Zhu, Y., Feng, Y., Wang, Y., Zhao, H., Zhao, N., Zhang, H., He, S., Liu, Q. and Xu, R., 2023. Fast track to obtain heritable transgenic sweet potato inspired by its evolutionary history as a naturally transgenic plant. *Plant Biotechnology Journal*, *21*(4), pp.671-673.

Zhang, Y.M., Liu, Z.H., Yang, R.J., Li, G.L., Guo, X.L., Zhang, H.N., Zhang, H.M., Di, R., Zhao, Q.S. and Zhang, M.C., 2016. Improvement of soybean transformation via *Agrobacterium* tumefaciens methods involving α-aminooxyacetic acid and sonication treatments enlightened by gene expression profile analysis. Plant cell reports, 35, pp.1259-1271.

Zia, M., Arshad, W., Bibi, Y., Nisa, S. and Chaudhary, M.F., 2011. Does Agro-injection to soybean pods transform embryos. *Plant Omics Journal*, *4*(7), pp.384-390.

VITA

1. Name of student : Zanjal Sagar Laxman
2. Date of Birth : 03 January 2000
3. Name of the College : Post Graduate Institute

   Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
4. Residential Address : At. Sawari post. Jawahar Nagar Mah,
   Tq. Bhandara Dist. Bhandara
   Maharashtra- 441906
5. Academic Qualification :

Sr. No.	Name of Degree awarded	Year in which obtained	Division/ Class	Name of awarding University	Subjects
1.	B.Sc. (Agril. Hons.)	2022	First Division	Dr. PDKV, Akola	Agriculture

6. Research papers published :
   (If any)
7. Field of Interest :
   (In which you desire to work)

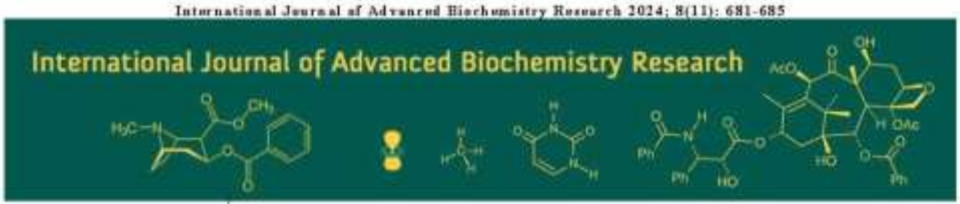
Place: Akola **Signature of Student**

Date : / /2024 **(Zanjal Sagar Laxman)**

Appendix

Thesis based research paper

Title	**:**	Establishment of ovary targeted *Agro*-injection method for *Inplanta* soybean transformation
Author	**:**	Sagar L Zanjal, Pravin V Jadhav, Gopika Mote, Arpita Mahobia, Ruchika Bhagat, Bhagyashri Gavande, MP Moharil, Dipika Padole and SS Nichal
Affiliation	**:**	Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.
Correspondence	**:**	@gmail.com
Journal	**:**	International Journal of Advance Biochemistry Research.
NAAS Rating	**:**	05.29
Citation	**:**	Sagar L Zanjal, Pravin V Jadhav, Gopika Mote, Arpita Mahobia, Ruchika Bhagat, Bhagyashri Gavande, MP Moharil, Dipika Padole and SS Nichal. Establishment of ovary targeted *Agro*-injection method for *Inplanta* soybean transformation. Int. J. Adv. Biochem. Res. 2024;8(11):681-685. DOI: 10.33545/26174693.2024.v8.i11i.2938
Enclosed	**:**	Research paper based on a thesis.



ISSN Print: 2617-4693
ISSN Online: 2617-4707
IJABR 2024; 8(11): 681-685
www.biochemjournal.com
Received: 26-08-2024
Accepted: 06-10-2024

Sagar L Zanjal
Biotechnology Centre, Post Graduate Institute, Department of Agriculture Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Pravin V Jadhav
Biotechnology Centre, Post Graduate Institute, Department of Agriculture Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Gopika Mote
Biotechnology Centre, Post Graduate Institute, Department of Agriculture Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Arpita Mahobia
Biotechnology Centre, Post Graduate Institute, Department of Agriculture Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Ruchika Bhagat
Biotechnology Centre, Post Graduate Institute, Department of Agriculture Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Bhagyashri Gavande
Biotechnology Centre, Post Graduate Institute, Department of Agriculture Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

MP Moharil
Biotechnology Centre, Post Graduate Institute, Department of Agriculture Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Dipika Padole
Biotechnology Centre, Post Graduate Institute, Department of Agriculture Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

SS Nichal
Regional Research Centre, Amravati, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Corresponding Author:
Sagar L Zanjal
Biotechnology Centre, Post Graduate Institute, Department of Agri. Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Establishment of ovary targeted *Agro*-injection method for *Inplanta* soybean transformation

Sagar L Zanjal, Pravin V Jadhav, Gopika Mote, Arpita Mahobia, Ruchika Bhagat, Bhagyashri Gavande, MP Moharil, Dipika Padole and SS Nichal

DOI: https://doi.org/10.33545/26174693.2024.v8.i11i.2938

Abstract

Soybean transformation using *Agro*-injection was analyzed by targeting the ovary at the floret stage. *Agrobacterium tumefaciens* EHA 105, harboring the Pdirect22A plasmid targeting the BADH2 gene, was injected across three genotypes: PDKV Amba, Swarna Vasundhara, and PDKV Yellow Gold. The objective was to evaluate its impact on pod development, seed germination, and transformation efficiency. Seeds obtained from *Agro*-injection were assessed for transformation efficiency based on the percentage of PCR-positive plants. The highest transformation efficiency was observed in PDKV Amba (2.67%), followed by PDKV Yellow Gold (1%), with Swarna Vasundhara showing the lowest efficiency (0.66%). The highest pod-bearing percentage was recorded in PDKV Amba (32.21%), followed by PDKV Yellow Gold (30.68%), while Swarna Vasundhara had a significantly lower rate (12.92%). In contrast, seed germination rates were highest in Swarna Vasundhara (96.25%), followed by PDKV Yellow Gold (95.17%), with PDKV Amba exhibiting the lowest germination rate (87.66%). These results demonstrate a clear genotype-specific response. PDKV Amba showed the most favorable outcomes in terms of both pod formation and transformation success. Meanwhile, PDKV Yellow Gold, despite a high number of flower injections, exhibited lower transformation efficiency. These findings suggest that although *Agro*-injection is a promising technique for soybean transformation, optimizing the protocol for specific genotypes is essential to maximize efficiency and reproducibility.

Keywords: Soybean, *in-planta* transformation, CRISPR/Cas9, SAAT, *Agro*-injection, gene editing, BADH2

Introduction

Genetic transformation has become a vital tool in modern crop improvement, enabling the direct introduction of valuable traits into important crops like soybean (*Glycine max* L. Merr.). This technology complements traditional breeding approaches, which often face limitations due to restricted genetic diversity. As a major global crop, soybean is prized for its high protein (40%) and oil (20%) content, making it essential for food, animal feed, and various industrial applications. However, soybean is known to be recalcitrant to transformation, with significant challenges arising from its strong genotype dependence and low efficiency in conventional transformation protocols (Bent, 2000; Mello-Farias and Chaves, 2008) [1, 11].

Traditional methods for introducing genes into soybean, such as *Agrobacterium tumefaciens*-mediated transformation, biolistic particle bombardment, and electroporation, have been commonly used. Among these, *Agrobacterium*-mediated transformation is preferred due to its ability to achieve stable gene integration with fewer insertions. However, this approach in soybean is limited by labour-intensive tissue culture steps that often result in low efficiency and high genotype specificity (Hansen and Wright, 1999; Mamidala and Nanna, 2009) [5, 10]. Alternative methods like the biolistic approach (Finer and McMullen, 1991) [3] and modified *Agrobacterium* techniques (Hinchee *et al.*, 1988; Olhoft *et al.*, 2001) [8, 12] have been explored but still face issues such as high chimerism rates and inconsistent results. The cotyledonary node method, while promising, is slow and heavily dependent on tissue culture, making it impractical for large-scale use (Olhoft *et al.*, 2001; Paz *et al.*, 2004) [12, 13].

To tackle these challenges, researchers have developed in planta transformation methods that skip tissue culture and simplify the process. Techniques like floral-dip and vacuum infiltration have shown success in model plants such as *Arabidopsis thaliana* (Clough and Bent, 1998) [2], but applying them to leguminous crops like soybean has been difficult. Early attempts, including flower infiltration, often produced inconsistent results due to challenges with stable gene integration (Li *et al*., 2002) [7]. However, Trieu *et al*. (2000) [15] managed to create stable transgenics in *Medicago truncatula* using seedling infection and flower infiltration with *Agrobacterium tumefaciens*. More recent advancements by Lu *et al*. (2009) [9] and Liu *et al*. (2009) [8] have improved transformation efficiency using ovary transformation and drip methods. Li *et al*. (2010) also successfully generated chimeric soybean plants by inoculating *Agrobacterium rhizogenes* into seedling vascular bundles. To build on these improvements, this study introduces the *Agro*injection method, which directly injects *Agrobacterium* into floret targeting ovary. This technique avoids complex tissue culture steps and aims to make soybean transformation more efficient and accessible. This study focuses on evaluating the effectiveness of *Agro-injection* as an *in-planta* transformation method specifically targeting the ovary in soybean. We aim to assess the impact of this method on pod development, seed germination, and transformation efficiency across different soybean genotypes. By optimizing the *Agro-injection* protocol, we seek to establish a more efficient, genotype-independent method for stable transgene integration in soybean, ultimately contributing to advancements in soybean genetic transformation and crop improvement.

Table 1: Primer sequence used of BADH2 genes and Cas9 for PCR amplification

Gene	Direction	Primer sequence	Band size
GmBADH2 gRNA	Forward	GTCTATGAATAACTGCCGAT	425 bp
	Reverse	CGAACGGATAAACCTTTTCACG	
Cas9	Forward	GGAACTTCGAAGAGGTGGTG	810 bp
	Reverse	CTTCACAGTCTGGAGGATTC	

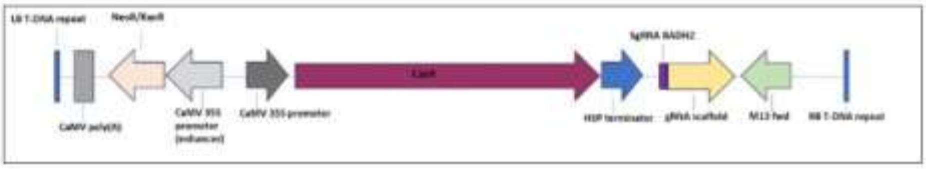


Fig 1: T-DNA region of transformed vector pDIRECT22A. LB T-DNA repeat=left Border, CaMV poly(A)=, NeoR/KanR=, CaMV 355 Promotor, Cas9, HSP terminator, SgRNA BADH2, gRNA scaffold, M13 fwd, RB T-DNA repeat=Right Border

Materials and Methods

Soybean cultivars

Three elite soybean genotypes with distinct agronomic characteristics were chosen for the study: Swarna Vasundhara a vegetable-type genotype with high yield potential from ICAR-RCER, Ranchi. Two grain-type variety AMS MB 5-18 (Swarna Soya) known for pod shattering resistance, and PDKV Yellow Gold resistant to charcoal rot and yellow mosaic virus provided by Dr. Panjabrao Deshmukh Krishi Vidyapeeth., sourced from Dr. Panjabrao Deshmukh Krishi Vidyapeeth.

Agrobacterium tumefaciens strain, vectors, and culture conditions

The *Agrobacterium* strain EHA 105 carrying the pDIRECT_22A plasmid vector, which harbors gRNA targeting the BADH2 genes (fig1), was maintained on solid LB medium supplemented with rifampicin (30 mg/l) and kanamycin (50 mg/l) incubated at 28±2 °C for 48 hours, and then preserved at 4°C. A single colony of *A. tumefaciens* was inoculated into 5 mL of Luria Broth (LB) supplemented with the rifampicin (30 mg/l) and kanamycin (50 mg/l) antibiotics and incubated overnight at 28±2 °C, under orbital shaker with 150 rpm the following day, 100 µl of the overnight-grown culture was transferred into 50 ml of LB broth supplemented with 50 µl of acetosyringone stock solution (40 mg/ml) in a 50 ml and incubated at 28±2 °C for 48 hours culture under orbital shaker with 150 rpm. And adjusted to a bacterial density concentration of 0.2 to 1.0 OD600 nm for transformation.

Fig 2: Injection of *Agrobacterium* to soybean flowers: (a) *Agro*-injection to floret of soybean (b) microscopy of soybean flower (c) target organ (d) pod development (e) maturation of pod f) PCR positive plant

In-planta Transformation Technique

Agro-Injection at floret

Referring to the transformation methods developed by Zia *et al*. (2011) [16] and Liu *et al*. (2009) [8], we evaluated the effectiveness of *Agro*-injection at the ovary to study its potential for achieving transformation in soybeans. For this, soybean seeds were sown in pots containing sterilized autoclaved soil under greenhouse conditions. At the flowering stage, flowers at the crossing stage were selected, and fresh *Agrobacterium* cultures (O.D. at 600 nm = 0.6) were prepared before each infection. The cultures were drawn into syringes and injected directly into the ovary region of the florets. Following injection, the flowers were tagged and labelled appropriately for identification.

The *Agro*-injection procedure was carried out in the morning hours, targeting flowers at the crossing stage. Untreated flowers and buds at the same node were carefully removed to minimize interference. Observations were recorded throughout the process. The seeds from the injected flowers were harvested and subsequently planted for analysis of transformation percentage. Additionally, seeds from untreated flowers at different nodes were collected and used as controls in the subsequent molecular analyses.

Selecting soybean transformants by PCR

The plants germinated of different genotypes treatments a were subjected to polymerase chain reaction to confirm the presence of inserted fragment of plasmid. Leaf was used for extraction of genomic using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson,1980) and Plasmid DNA was isolated from transformed *Agrobacterium* using alkaline lysis method of giving by (Warburton, 2205) [14]. PCR was performed using BADH2 genes and Cas9. The primer sequences used for BADH2 and Cas9 are given in table 1. The PCR reaction mixture (10 µl) contained 2x Master Mix (EmeraldAmp GT PCR Master Mix), NFW(Nuclease free water) (3 µl), forward primer (10 pmol), reverse primer (10 pmol), template DNA (100 ng total genomic DNA, or 10 pg plasmid DNA). The PCR reaction was conducted using an initial denaturation at 94 °C for 5 min followed by 32 cycles of 94 °C for 30 s, 48 °C (for badh2) and 54°C (for Cas9) for 30 s, 72 °C for 45 s, and a final extension of 10 min at 72 °C. For a positive PCR control, plasmid DNA isolated from the transformed Agrobacterium colony habouring sgRNA targeting BADH2 was used as a template. The PCR products were analysed on 2.0% agarose gels with the expected product size of 810bp for Cas9 specific primer and 425bp for BADH2 specific primer.

Table 2: Analysis of Seed Germination, Pod Development, and Transformation Efficiency in Soybean Genotypes Following Embryo Stage *Agro-injection*

Genotypes	Flowers injected	Pods developed	Pod bearing %	Seeds harvested	Seed germinated	Germination percentage	PCR positive		Total PCR positive	Transformation efficiency
							BADH2	Cas9		
PDKV Amba	239	77	32.21	154	112	72.7	1	2	3	2.67
Swarna Vasundhara	936	121	12.92	240	151	62.9	0	1	1	0.66
PDKV Yellow Gold	1007	309	30.68	643	498	77.4	3	2	5	1

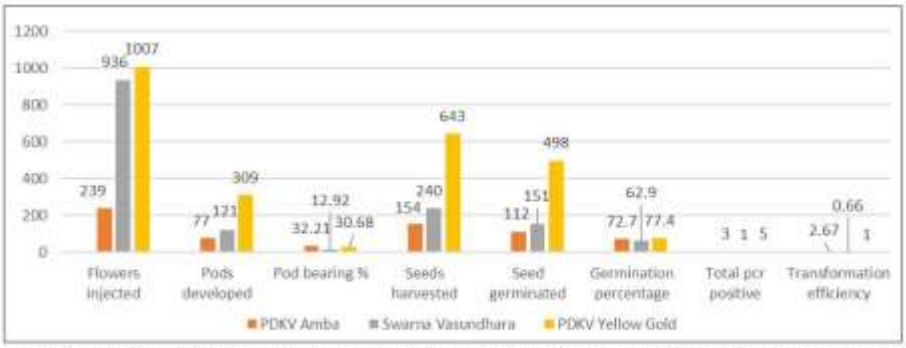


Fig: Graphical presentation of effect on Seed Germination, Pod Development, and Transformation Efficiency in Soybean Genotypes of *Agro*-injection method.

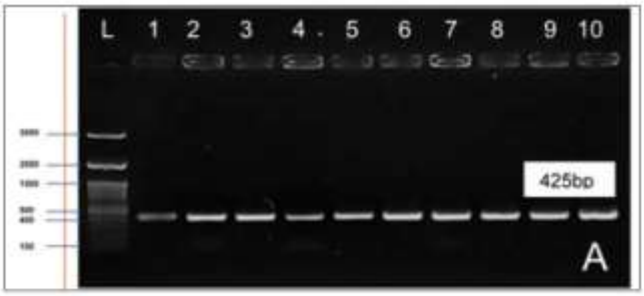


Fig 4: Gel picture of PCR positive plants and confirmation of transformed *Agrobacterium* using plasmid DNA A)L-Ladder 100bp,1-10 PCR positive *Agrobacterium* plasmid for gRNA specific primer BADH2 gene B) Ladder 100bp,1-3 PCR positive for Cas9 specific primer,4-control,6-7 Positive control 8-11 PCR positive for sgRNA specific primer, 12-Control, 14,15-Postive control

Results

Effect of *Agro*-injection on Pod Bearing Rate

The impact of *Agro*-injection on pod development was assessed across three soybean genotypes: PDKV Amba, Swarna Vasundhara, and PDKV Yellow Gold. PDKV Amba exhibited the highest pod bearing percentage at 32.21%, followed by PDKV Yellow Gold with a pod bearing rate of 30.68%. In contrast, Swarna Vasundhara demonstrated a markedly lower pod bearing percentage of 12.92%. These findings indicate that the efficacy of *Agro*-injection in enhancing pod set is genotype-dependent, with PDKV Amba and PDKV Yellow Gold showing a favourable response, while Swarna Vasundhara exhibited limited pod formation despite a higher number of flowers injected.

Effect of Agro-injection on Seed Germination Rate

The germination performance of seeds obtained from *Agro*-injection treated plants varied among the genotypes. PDKV Yellow Gold showed the highest seed germination rate at 77.4%, indicating minimal adverse effects on seed viability. PDKV Amba followed with a germination rate of 72.7%, while Swarna Vasundhara exhibited the lowest germination rate of 62.9%. The reduced germination rate observed in Swarna Vasundhara may suggest a potential genotype-specific sensitivity to the *Agro*-injection process, affecting seed viability.

Influence of *Agro*-injection on Transformation Efficiency

In this study, each PCR-positive plant was considered a single transformation event to calculate transformation efficiency determined by the percentage of PCR-positive plants among the total seeds germinated, varied across the genotypes obtaining nine PCR positive. In the PDKV Amba genotype, two PCR-positive plant was identified using the Cas9-specific primer, and one PCR-positive plant was found using the sgRNA-specific primer with highest transformation efficiency at 2.67%, indicating its favourable response to Agro-injection-mediated gene transfer. In the PDKV Yellow Gold genotype, two PCR-positive plant was identified using the Cas9-specific primer, and three PCR-positive plant was found using the sgRNA-specific primer with a transformation efficiency of 1.00%, while Swarna Vasundhara recorded the lowest efficiency at 0.66%. These results highlight that transformation efficiency is not solely correlated with flower injection frequency or seed yield but is also influenced by genotype-specific factors that may affect gene transfer and integration efficiency.

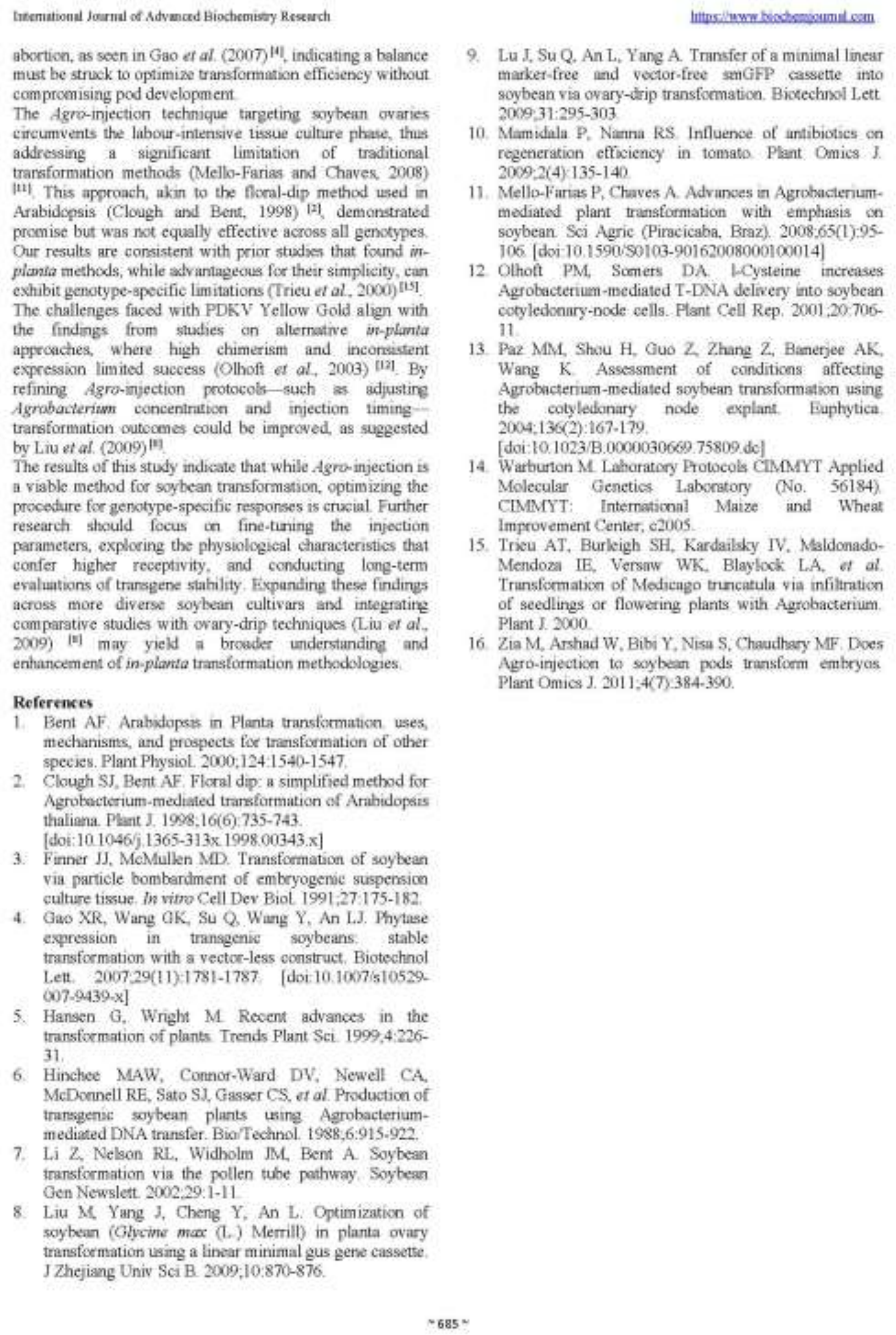
Discussion

The results from our study demonstrate the potential of *Agro*-injection as an in-planta transformation method for soybean, showing significant genotype-specific variations in pod development, seed germination, and transformation efficiency. Among the tested genotypes—PDKV Amba, Swarna Vasundhara, and PDKV Yellow Gold—PDKV Amba exhibited the highest transformation efficiency, followed by PDKV Yellow Gold and Swarna Vasundhara. These findings underscore the inherent genotype-dependent responses observed in soybean transformation methods, which have been a consistent challenge in plant biotechnology (Hansen and Wright, 1999; Bent, 2000) [5, 1].

Our data revealed that PDKV Amba had the highest transformation efficiency (2.67%) and pod bearing rate (32.21%), suggesting its suitability for *Agro*-injection-based transformation. This aligns with findings from Liu *et al.* (2009) [8], who reported enhanced transformation outcomes with optimized in-planta methods targeting reproductive tissues. Conversely, PDKV Yellow Gold, despite achieving the highest seed germination rate (77.4%), demonstrated a relatively lower transformation efficiency (1.00%). This observation highlights that while seed viability and pod development are critical, they do not directly correlate with transformation success.

In comparison, the study by Zia *et al.* (2011) [16] using *Agrobacterium*-mediated pod injection demonstrated variability in transformation efficiencies depending on the stage of pod development. For instance, treating pods at early stages yielded significant GUS expression in seeds and cotyledons but reduced overall plant transformation rates due to embryo damage. Our findings echo this pattern, with early and targeted *Agro*-injection yielding differential responses across genotypes. PDKV Yellow Gold, which had a high number of treated flowers with transformation efficiency (1.00%), reflects the challenges noted by previous researchers in achieving consistent outcomes across different soybean varieties (Olhoft *et al.*, 2001) [12].

The strong seed germination performance observed in PDKV Yellow Gold may indicate that genetic or physiological traits promoting seed viability do not necessarily enhance the integration and expression of transgenes. This dissociation between seed viability and transformation rates suggests that factors such as *Agrobacterium* susceptibility, timing of injection, and tissue receptivity play pivotal roles. Liu *et al.* (2009) [8] emphasized that modifications to the floral pathway, such as ovary cutting, improved DNA uptake and reduced chimerism. However, excessive manipulation risks pod



abortion, as seen in Gao *et al.* (2007) [4], indicating a balance must be struck to optimize transformation efficiency without compromising pod development.

The *Agro*-injection technique targeting soybean ovaries circumvents the labour-intensive tissue culture phase, thus addressing a significant limitation of traditional transformation methods (Mello-Farias and Chaves, 2008) [11]. This approach, akin to the floral-dip method used in Arabidopsis (Clough and Bent, 1998) [2], demonstrated promise but was not equally effective across all genotypes. Our results are consistent with prior studies that found *in-planta* methods, while advantageous for their simplicity, can exhibit genotype-specific limitations (Trieu *et al.*, 2000) [15].

The challenges faced with PDKV Yellow Gold align with the findings from studies on alternative *in-planta* approaches, where high chimerism and inconsistent expression limited success (Olhoft *et al.*, 2003) [12]. By refining *Agro*-injection protocols—such as adjusting *Agrobacterium* concentration and injection timing—transformation outcomes could be improved, as suggested by Liu *et al.* (2009) [8].

The results of this study indicate that while *Agro*-injection is a viable method for soybean transformation, optimizing the procedure for genotype-specific responses is crucial. Further research should focus on fine-tuning the injection parameters, exploring the physiological characteristics that confer higher receptivity, and conducting long-term evaluations of transgene stability. Expanding these findings across more diverse soybean cultivars and integrating comparative studies with ovary-drip techniques (Liu *et al.*, 2009) [8] may yield a broader understanding and enhancement of *in-planta* transformation methodologies.

References

1. Bent AF. Arabidopsis in Planta transformation. uses, mechanisms, and prospects for transformation of other species. Plant Physiol. 2000;124:1540-1547.
2. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 1998;16(6):735-743. [doi:10.1046/j.1365-313x.1998.00343.x]
3. Finner JJ, McMullen MD. Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. *In vitro* Cell Dev Biol. 1991;27:175-182.
4. Gao XR, Wang GK, Su Q, Wang Y, An LJ. Phytase expression in transgenic soybeans: stable transformation with a vector-less construct. Biotechnol Lett. 2007;29(11):1781-1787. [doi:10.1007/s10529-007-9439-x]
5. Hansen G, Wright M. Recent advances in the transformation of plants. Trends Plant Sci. 1999;4:226-31.
6. Hinchee MAW, Connor-Ward DV, Newell CA, McDonnell RE, Sato SJ, Gasser CS, *et al.* Production of transgenic soybean plants using Agrobacterium-mediated DNA transfer. Bio/Technol. 1988;6:915-922.
7. Li Z, Nelson RL, Widholm JM, Bent A. Soybean transformation via the pollen tube pathway. Soybean Gen Newslett. 2002;29:1-11.
8. Liu M, Yang J, Cheng Y, An L. Optimization of soybean (*Glycine max* (L.) Merrill) in planta ovary transformation using a linear minimal gus gene cassette. J Zhejiang Univ Sci B. 2009;10:870-876.
9. Lu J, Su Q, An L, Yang A. Transfer of a minimal linear marker-free and vector-free smGFP cassette into soybean via ovary-drip transformation. Biotechnol Lett. 2009;31:295-303.
10. Mamidala P, Nanna RS. Influence of antibiotics on regeneration efficiency in tomato. Plant Omics J. 2009;2(4):135-140.
11. Mello-Farias P, Chaves A. Advances in Agrobacterium-mediated plant transformation with emphasis on soybean. Sci Agric (Piracicaba, Braz). 2008;65(1):95-106. [doi:10.1590/S0103-90162008000100014]
12. Olhoft PM, Somers DA. l-Cysteine increases Agrobacterium-mediated T-DNA delivery into soybean cotyledonary-node cells. Plant Cell Rep. 2001;20:706-11.
13. Paz MM, Shou H, Guo Z, Zhang Z, Banerjee AK, Wang K. Assessment of conditions affecting Agrobacterium-mediated soybean transformation using the cotyledonary node explant. Euphytica. 2004;136(2):167-179. [doi:10.1023/B.0000030669.75809.dc]
14. Warburton M. Laboratory Protocols CIMMYT Applied Molecular Genetics Laboratory (No. 56184). CIMMYT: International Maize and Wheat Improvement Center; c2005.
15. Trieu AT, Burleigh SH, Kardailsky IV, Maldonado-Mendoza IE, Versaw WK, Blaylock LA, *et al.* Transformation of Medicago truncatula via infiltration of seedlings or flowering plants with Agrobacterium. Plant J. 2000.
16. Zia M, Arshad W, Bibi Y, Nisa S, Chaudhary MF. Does Agro-injection to soybean pods transform embryos. Plant Omics J. 2011;4(7):384-390.