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Mahima Parihar
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Pravin Jadhav
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Umesh Shinde
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Namrata Thakare
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Mangesh Moharil
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

SS Nichal
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

SS Mane
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Corresponding Author:
Pravin Jadhav
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra, India

Optimization of sonication-assisted *Agrobacterium*-mediated transformation for enhanced explant recovery and regeneration in soybean (*Glycine Max* L.)

Mahima Parihar, Pravin Jadhav, Umesh Shinde, Namrata Thakare, Mangesh Moharil, SS Nichal and SS Mane

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Abstract
This study optimizes a sonication-assisted *Agrobacterium*-mediated transformation protocol for the soybean genotype PDKV Amba. The research aimed to efficiently deliver the pDIRECT_22A vector, which targets the β-conglycinin allergen using CRISPR/Cas9 technology. Evaluated co-cultivation durations of 2, 4, and 6 days following a 30-second sonication treatment at 50Hz. While the 2 day period showed the highest initial germination (92%) and the 6 day period yielded maximum survival on selection media (90.47%), the 4 day co-cultivation proved optimal. It achieved the highest overall recovery rate (39.07%) and produced the most viable plants, successfully balancing *Agrobacterium* infection with explant viability.

Keywords: Soybean, crispr, β-conglycinin, Agrobacterium, transformation

Introduction
Soybean (*Glycine max* L. Merr.) is one of the most important crop plants, accounting for a large share of the world market in oil crops and serving as an important source of plant-based proteins for both humans and as fodder. In 2025-26, more than 427.7 million metric tonnes of soybeans were produced on more than 146.8 million hectares worldwide. The seeds of soybean contain about 35-40% protein, 18-20% oil (Jadhav *et al*.,2025) [4], and are therefore utilised in a wide range of products for multiple uses. Though soybean is one of the most important crop species, the narrow genetic base or narrow range of genetic diversity, available for certain desired traits and the extended duration required for traditional breeding present considerable challenges in conventional strategies in soybean improvement. Soybean transformation surpasses traditional soybean breeding. It plays an important role in addressing the key issues related to soybean; despite the developments and refinement of the soybean transformation system over the past years remains less efficient compared to other crops. Due to the recalcitrant nature of the crop, with low transformation efficiency, prolonged tissue culture requirements, and frequent chimeric plant formation (Homrich *et al*., 2012; Altpeter *et al*., 2016) [1, 3]. An efficient and stable genetic transformation method is a prerequisite for successful transformation. In the current study, the emergence of genome-editing technologies has provided new opportunities for precise and targeted crop improvement. *Agrobacterium-mediated* transformation is very efficient for the delivery of targeted genes in plants because of its simplicity, efficiency, accuracy, and potential for multiplex gene editing.

Material and methods
Plant material:
High yielding, high protein content, Charcoal rot resistant soybean genotype PDKV Amba (AMS-100-39) was used for the genetic transformation.

Agrobacterium strain and vector

The *Agrobacterium* strain EHA 105 carries the pDIRECT_22A plasmid vector. The pDIRECT_22A contains kanamycin as a selectable marker gene and sgRNA targeting the subunits of the β-Conglycinin allergen.

Culture, explant preparation and agro-infection

Agrobacterium culture harbouring sgRNA targeting the subunits of the β-Conglycinin allergen was cultured on liquid LB medium with rifampicin (30 mg/l) and kanamycin (50 mg/l) at 28±2°C, at 150 rpm. Then the culture was transferred into 50 mL of MS medium supplemented with 50 µl of Acetosyringone (40 mg/ml), and incubated at 28 °C for 30 min before use in explant infection. The inoculum density was adjusted to OD600nm=0.6. Soybean (Glycine max. (L.) Merr.) seeds from PDKV-Amba genotypes were imbibed in sterile water overnight. After soaking, the seeds were surface sterilized with Tween® 20, then with 70% ethanol for 30 sec, followed by 1% fungicide for 10 min, and finally with 0.1% $HgCl_2$ for 30 sec at each surface sterilization step washing with sterile water 2-3 times. Under the flow hood, the surface-sterilised seeds were transferred to a sterile glass plate and, using a scalpel blade, a longitudinal cut along the hilum was made to separate the cotyledons (Fig.2). The seed coat was removed, and the embryonic axis found at the nodal end of the cotyledons was excised also; any remaining axial buds attached to the cotyledonary node were removed, and these half-seed explants were transferred to liquid agro-inoculation. The sonication-mediated agro-infection approach was employed at 50Hz frequency for 30 sec.

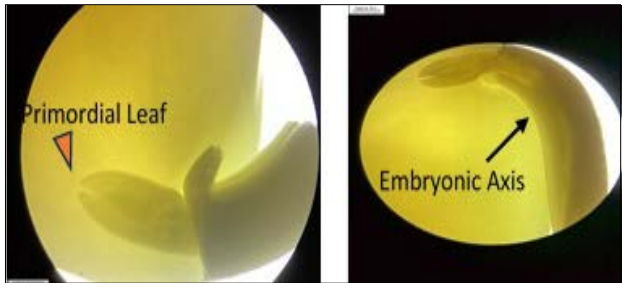

Fig 1: Explant preparation and site of excision

After the Agrobacterium infection, the explants were placed with adaxial sides up on a liquid co-cultivation medium to initiate germination under controlled conditions for 2 days, 4 days and 6 days. This step is crucial to minimising bacterial overgrowth and reducing tissue damage caused by excess moisture. Following co-cultivation, the explants were transferred to a pre-selection medium to facilitate recovery and support their initial development. The pre-selection medium consisted of Murashige and Skoog (MS) basal medium supplemented with MES buffer (0.58 g/L), cefotaxime (100 mg/L), 6BAP (1.5mg/L), and GA_3 (0.25 mg/L). The medium was solidified with 0.6% (w/v) agar and adjusted to pH 5.8 prior to autoclaving. The cultures were incubated at 25 °C under a light intensity of 160-180 µmol m^{-2} s^{-1} with a 16 h light/8 h dark photoperiod for 5 days. Following pre-selection, the infected germinated seedlings were transplanted into soil for additional selection and growth of shoots (Fig.3)

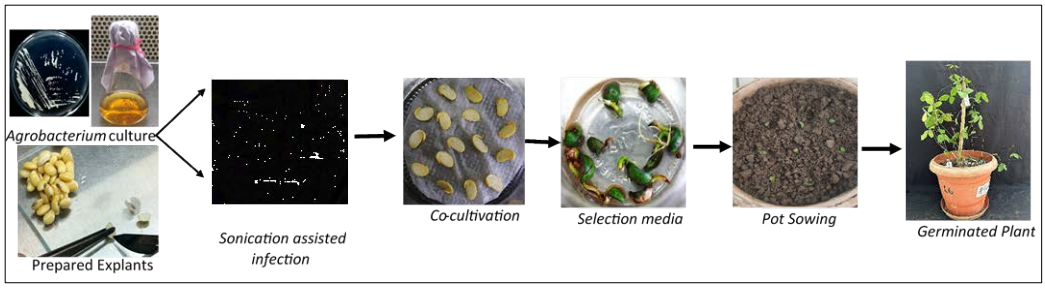

Fig 2: Agro-infection pipeline

Result

The genetic transformation in soybean faces challenges. Like genotype-dependent regeneration, low transformation efficiency, stress from tissue culture and poor survival of regenerated plants. To address these issues, an optimised transformation and regeneration protocol was evaluated. Careful monitoring was done at each step: germination, seedling establishment and plant survival. Different co-cultivation durations were tested with sonication-assisted treatments.

Effect of co-cultivation duration on explant regeneration and survival

The effect of co-cultivation duration on explant regeneration following sonication-assisted transformation was evaluated. The highest germination response was observed with a 2-day co-cultivation period, during which 92.0% of the explants exhibited germination. Co-cultivation for 4 and 6 days resulted in germination rates of 85.50% and 85.40%, respectively. Although the germination percentages for the 4- and 6-day treatments were comparable, both were lower than that recorded after 2 days of co-cultivation. Thus, based on the initial germination response, the 2-day co-cultivation treatment exhibited the highest regeneration potential under the sonication-assisted liquid co-cultivation conditions (Table 1).

Survival of explants on selection i medium

Following co-cultivation, the explants were transferred to Selection I medium containing kanamycin to facilitate the selection of putatively transformed tissues. The survival of explants varied considerably with co-cultivation duration. Following 2 days of co-cultivation, 94 of 138 explants survived on Selection I medium, corresponding to a survival rate of 68.11%. In the 4-day co-cultivation treatment, 151 of 171 explants survived, resulting in a survival rate of 80.30%. The highest survival rate was recorded following 6 days of co-cultivation, with 90.47% of explants surviving on Selection I medium (Table 1).

The lower survival observed following the shorter co-cultivation treatment may be associated with the effects of sonication and subsequent selection stress. Sonication

facilitates Agrobacterium-mediated transformation by generating transient micro-wounds that may enhance bacterial attachment and T-DNA delivery into plant tissues. However, excessive ultrasound exposure can result in cellular and tissue damage, potentially reducing explant viability. Santarém *et al.* (1998) [7]. reported that sonication enhanced Agrobacterium-mediated infection in soybean, while prolonged sonication increased tissue damage. Similarly, studies involving different soybean cultivars have demonstrated that appropriately optimized sonication conditions can enhance transformation efficiency, whereas excessive treatment intensity may adversely affect explant health. These observations indicate that the effectiveness of sonication is dependent on the balance between enhanced bacterial infection and preservation of explant viability.

Recovery and regeneration response

The recovery response of explants following selection differed among the co-cultivation treatments. The highest recovery rate was observed with the 4-day co-cultivation treatment (39.07%), whereas substantially lower recovery rates were recorded for the 2-day (11.70%) and 6-day (18.79%) treatments. Although the 2-day treatment resulted in the highest initial germination percentage and the 6-day treatment produced the highest survival on Selection I medium, the 4-day treatment demonstrated the highest recovery rate following selection. Considering the overall regeneration response and recovery percentage, the 4-day co-cultivation period was selected as the optimum duration for the subsequent optimization of the sonication-assisted transformation protocol in soybean explants. This treatment provided a comparatively favourable balance between explant survival and post-selection recovery and was therefore used for further optimization experiments.

Table 1: Effect of sonication-assisted treatments of soybean following co-cultivation duration

Stage	2-day Sonication	4-day Sonication	6-day Sonication	Total
No. of explants used for co-cultivation	150	200	172	522
No. of explants in Selection I	138	171	147	456
No. of shoots under sowing	94	151	133	378
No. of plants raised	11	59	25	95
Germination (%)	92 %	85.50 %	85.40 %	87.35%
Survival rate (%)	68.11%	88.30%	90.47%	82.89%
Recovery (%)	11.70%	39.07%	18.79%	25.13%

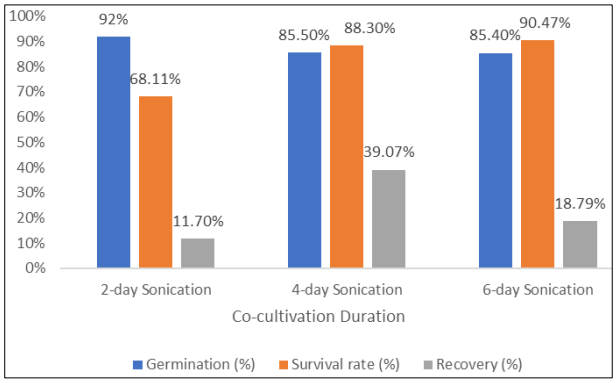

Fig 3: The effect of Cocultivation duration on germination, survival and recovery of soybean.

Discussion

The response observed in the present study may be related to the physical effects associated with sonication-assisted treatment. Ultrasound treatment generates transient mechanical disturbances and micro-wounds in plant tissues, which can facilitate *Agrobacterium* attachment and T-DNA delivery. However, excessive physical stress may cause cellular damage and adversely affect subsequent tissue recovery and regeneration. Santarém *et al.* (1998) [8] reported that sonication could enhance *Agrobacterium*-mediated infection in soybean while also demonstrating the potential for increased tissue damage with prolonged or excessive sonication. The present results are consistent with the concept underlying Sonication-Assisted *Agrobacterium*-mediated Transformation (SAAT) described by Trick and Finer (1997) [8], in which controlled wounding is used to enhance transformation while minimizing damage to the target tissue. The response observed in PDKV Amba also complements the cotyledonary-node transformation approaches reported by Paz *et al.* (2006) [6], highlighting the importance of optimizing physical treatment conditions in soybean transformation systems. The relatively higher recovery obtained after 4 days of co-cultivation is consistent with reports indicating that extending the co-cultivation period beyond the commonly used 2-day period can enhance the interaction between *Agrobacterium* and soybean tissues under appropriate experimental conditions. However, the optimum duration is dependent on several factors, including explant type, soybean genotype, *Agrobacterium* strain, bacterial density, infection conditions, and composition of the culture medium. Li *et al.* (2017) reported efficient infection of soybean half-seed cotyledonary explants following a 5-day co-cultivation period, although their experimental conditions differed from those employed in the present study. Their findings also demonstrated that successful transformation depends not only on *Agrobacterium* infection but also on the subsequent ability of the transformed tissue to survive selection and regenerate into shoots and complete plants. The substantially lower recovery observed after 2 days of co-cultivation (11.70%) may indicate that the shorter interaction period was insufficient to achieve an optimal balance between *Agrobacterium* infection and subsequent tissue recovery under the conditions used in the present study. Conversely, although the 6-day treatment resulted in the highest survival rate on Selection I medium (90.47%), it did not produce the highest recovery or regeneration response. This observation demonstrates that explant survival alone cannot be considered a direct measure of transformation or regeneration efficiency. Explants may remain viable under selection conditions without subsequently developing shoots or regenerating into complete plants. Therefore, evaluation of transformation protocols should consider multiple parameters, including survival, recovery, shoot induction, regeneration, and ultimately the production of putatively

transformed plants. Co-cultivation duration is therefore an important variable in *Agrobacterium*-mediated transformation. An adequate period is required to permit bacterial attachment, infection, and T-DNA transfer, whereas excessive co-cultivation may increase bacterial proliferation and impose additional stress on the explant. In the present study, the 4-day co-cultivation treatment produced the highest recovery response and the greatest number of plants raised among the treatments evaluated. These findings indicate that, for PDKV Amba under the experimental conditions used, a 4-day co-cultivation period provided a favourable balance between *Agrobacterium* interaction and explant recovery following sonication-assisted treatment. Further optimization of both co-cultivation duration and sonication parameters may therefore improve the efficiency and reproducibility of *Agrobacterium*-mediated transformation in this soybean genotype.

Conclusion

The present study demonstrated that co-cultivation duration influenced explant recovery and regeneration in sonication-assisted Agrobacterium-mediated transformation of soybean genotype PDKV Amba. Among the treatments tested, 4-day co-cultivation resulted in the highest recovery (39.07%) and the highest number of plants raised. Although 6-day co-cultivation showed higher explant survival, it did not improve overall recovery, highlighting the importance of balancing Agrobacterium interaction with explant viability. The optimized conditions provide a preliminary basis for developing an Agrobacterium-mediated delivery system for the pDIRECT_22A construct targeting the β-conglycinin trait and may support future CRISPR/Cas9-based improvement of soybean.

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Data availability

Data will be made available on request

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