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Transgenic sunflower genotypes: development, evaluation, and improvement strategies

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Abstract: Sunflower varieties have been developed through genetic manipulation, aiming to enhance various traits for improved agronomic performance and product quality. However, an industrial technology specifically for sunflower breeding programs to develop transgenic genotypes in a more feasible way has not yet been integrated into the sunflower research programs. In this study we present our endeavor of obtaining transgenic sunflower genotypes, using Agrobacterium strains EHA105 and LBA440 in conjunction with two binary vectors, pROK2_35S-int-GUS and pTCO272_355-Int GUS. Bacterial strains were cultivated in liquid YEB medium supplemented with 2 mM $MgCl_2$ or $MgSO_4$ and antibiotics. Following bacterial propagation and centrifugation, the resulting cell pellet was resuspended in a solution containing 5% sucrose, 10µl/l BAP, and 0.01% Silwet L-77. Sunflower flower heads of varying sizes were immersed in this bacterial suspension for 2-4 minutes, followed by covering with aluminum foil to maintain humidity for 24 hours. The results of the study highlighted the importance of selecting the right moment for immersing the bacterial suspension and the stage of capitulum development in sunflowers to maximize the rate of obtaining transgenic genotypes. Thus, identifying an optimal period for this process in sunflowers is crucial to achieving a higher rate of transgenic plants.

Keywords: Transgenic sunflower varieties, *Agrobacterium* strains, EHA105 and LBA440 strains;

Introduction

Sunflower (*Helianthus annuus, 2n=34*) is one of the most important oilseeds crops globally due to its high-quality oil [1]. Sunflower seed production is influenced by climatic conditions, soil type, and applied cultivation technology [2], [3]. Globally, Ukraine is a top producer, with an annual production of 15-17 million tons, cultivated on approximately 6 million hectares. In Europe, the annual production is about 9-10 million tons, cultivated on approximately 4.5 million hectares, with Romania, Bulgaria, Hungary, and France being the main producers. Argentina also presents significant production, with an annual yield of 3-4 million tons on approximately 1.8 million hectares [4], [5].

The absence of transgenic sunflower genotypes on the market and the delay in the acceptance of genome editing tools, such as CRISPR/Cas9 technology, only serve to inhibit the acceptance and study of applicable technologies for large-scale sunflower breeding programs [6]. Altough, sunflower breeding programs are evolving increasingly [7].

In the early 19th century conventional methods and sunflower varieties were still used [8], today the creation and utilization of sunflower hybrids have become common. Biotechnology is employed to increase the number of generations and even new breeding methods are being developed to reduce the number of inbred lines in breeding programs [9], [10].

Biotechnology is significantly influencing sunflower breeding through genetic selection and improvement, using genes for disease resistance and drought tolerance [11].

The use of biotechnology plays an essential role in maximizing the cultivation area and creating valuable genotypes with increased tolerance to pests and pathogens, which significantly affect sunflower seed production [12]. Among the pathogens with the most significant impact on production are *Orobanche cumana* [13] and *Plasmopara halstedii* [14].

Regarding the development of transgenic genotypes resistant to *Orobanche cumana* [15], it is necessary, from the perspective of genetic editing implementation strategies, to avoid editing resistance mechanisms that include the germination of the *Orobanche cumana* parasite [16].

This is because the genetic variability of the parasite will eventually counteract the genetic diversity induced by genetic editing. On the other hand, it would be more effective to use genetic editing for mechanisms that investigate the role of sesquiterpene lactones (STLs) from sunflower in inhibiting the germination of the *Orobanche cumana* parasite [17]. Additionally, focusing on post-haustorial resistance systems that include editing could provide a more prolonged and durable resistance mechanism [18], [19].

The development of transgenic sunflower genotypes represents a substantial advancement in agricultural biotechnology [20], offering innovative solutions for improving resistance to these pathogens [21]. The development of transgenic sunflower genotypes begins with the identification and isolation of target genes that confer advantageous traits, such as resistance to herbicides [22], pests, or diseases, improvement in oil quality, or tolerance to environmental stresses [23]. These genes are introduced into the sunflower genome through various transformation techniques, including *Agrobacterium*-mediated transformation [24]. The success rate of these transformations depends on several factors, including the choice of promoters, the design of the genetic construct, and the efficiency of the transformation protocol.

The obtained transgenic sunflower genotypes undergo rigorous evaluations to assess their performance and stability [25]. This process includes molecular analyses to confirm the presence and expression of transgenic genes, as well as phenotypic evaluations to determine the impact of these genes on agronomic traits [26].

The introduction of CRISPR/Cas9 gene editing technologies allows precise modifications of the sunflower genome, facilitating targeted improvements without introducing foreign DNA [27]. Thus, by genetically editing areas of interest for genetic resistance, whether for diseases, herbicides, or even pests, the new genotypes can overcome numerous obstacles and improve conventional breeding practices [28].

Given the rapid evolution of pathogens that require complex genes, necessitating the introgression of genes in both parental forms for the creation of sunflower hybrids, a feasible biotechnological method for sunflower breeding is necessary in the context of the pathogen for which resistance is sought [29], [30].

In this study, we aimed to present a simple method for obtaining transgenic genotypes using *Agrobacterium* strains, considering the increasing development of genetic editing in other crops, which could facilitate the large-scale introduction of the CRISPR/Cas9 complex in obtaining transgenic sunflower genotypes.

Material and Method

Germplasm description

For this study, the inbred line HA-291, obtained from the USDA gene bank, was utilized. The HA-291 line has a flowering period that ranges from 55 to 64 days after planting. The head shape is predominantly flat, with a back slope sufficient to drain water. The capitulum diameter varies between 13 and 23 cm, and the total stem length ranges from 40 cm to 152 cm, depending on the growth conditions. In terms of productivity, HA-291 exhibits an oil content of 36% of the seed's dry weight. The weight of 100 seeds varies between 4.8 and 7.3 grams.

Description of Agrobacterium strains

For this study strains of *Agrobacterium* were used, EHA105 and LBA440. EHA105 is a hypervirulent strain of *Agrobacterium tumefaciens* derived from the strain A281. It is commonly used because of its high efficiency in transferring T-DNA into plant cells, making it a popular choice for plant transformation. LBA440 a strain of *Agrobacterium tumefaciens*, often used in plant transformation derived from the octopine type strain and is known for its stability and reliability in genetic transformation procedures [31], [32].

Strains propagation condition and vector

For the cultivation of bacterial strains, a liquid YEB medium was used (0.5% Bacto-peptone, 0.1% yeast extract, 0.5% beef extract, 0.5% sucrose, pH: 7.0), freshly supplemented with 2mM $MgCl_2$ or $MgSO_4$ and appropriate antibiotics. Ca vectori am utilizat pROK2_35S-int-GUS si pTCO272_355-Int GUS. For the pROK2 vector, 100mg/l Kanamycin was used, and for the pTCO272 vector, 100mg/l Spectinomycin was used.

After 24 hours of bacterial propagation, the culture was centrifuged. Following centrifugation, a resuspension medium containing 5% sucrose, 10µl/l BAP, and 0.01% Silwet L-77 was added [33].

Sunflower growing conditions and transformation method

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The seeds were germinated in the automated rhizotron at the Biological Research Centre (BRC) in Szeged. The sunflower plants were grown under controlled conditions at 20-22°C with a photoperiod of 12 hours day / 12 hours night, until the appearance of the floral bud.

Figure 1. Steps of Genetic Transformation Using *Agrobacterium tumefaciens* Strains: (A) - Selection of capitula based on experimental variants, (B) - Immersion of sunflower capitula in the solution, (C) - Covering with aluminum foil.

The immersion of sunflower capitula selected (figure 1, A) of various sizes was done in this bacterial suspension for 2-4 minutes (figure 1, B), followed by covering them with aluminum foil (figure 1, C) to maintain humidity for 24 hours. Immersion was carried one to three times, from the appearance of the floral bud before flowering, to ensure genetic transformation.

Experimental design

The experiment was conducted at various stages of vegetative development, based on the size of the floral bud, leading to the following experimental variants:

- V_1- capitulum size of 1.6-1.9 cm
- V_2- capitulum size of 2.0-2.2 cm
- V_3- capitulum size of 2.5-2.7 cm
- V_4- capitulum size of 2.8-3.0 cm

Immersion was performed on three consecutive days to enhance the success rate of generating transgenic plants. The experiment was carried out in five repetitions for each experimental variant.

GUS assay method

In order to perform the GUS assay, seeds of newly transformed plants were collected from each variant and each repetition. The GUS staining solution was prepared by mixing the following components: 50 mM Sodium phosphate buffer (pH 7.0), 0.1% Triton X-100, 2 mM Potassium ferricyanide, 2 mM Potassium ferrocyanide, and 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide).

The seeds were then immersed in the prepared GUS staining solution. The samples were incubated at 37°C for 12-24 hours in the dark to allow the reaction to occur.

After incubation, the reaction was stopped by immersing the tissues in 70% ethanol. To clear the chlorophyll, repeated changes of 70% ethanol were performed until the tissue was adequately decolorized. The presence of GUS activity will be indicated by a blue color in the seed, confirming the expression of the GUS reporter gene.

Statistical analysis

In the statistical analysis, RStudio software v4.0.2 was used to perform the one-way ANOVA test. Tukey's test was used to compare the means among the variants regarding the number of resulting transgenic plants and to determine the existence of statistically significant differences. Additionally, polynomial analysis was performed to investigate the relationship between the rate of transgenic plants obtained and the developmental stage of the caladium. All analyses were conducted at a significance level of $p < 0.05$.

Results and Discussion

Following the application of the genetic transformation methodology using *Agrobacterium tumefaciens* strains EHA105 and LBA440 at different stages of reproductive apparatus development, the importance of selecting the optimal period for obtaining transgenic plants was highlighted.

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The analysis of the percentage of transgenic plants obtained in relation to the developmental stage of the sunflower capitulum significantly influenced the rate of transgenic plant production ($p < 0.001$) (tabel 1).

Table 1. ANOVA Analysis of the Influence of Vegetation Period on the Percentage of Transgenic Plants

Variance source	Sum of squares	Df	Mean square	F	Sig.
Between groups	265,539	3	88,513	43,055	$p < 0,001$
Within groups	32,893	16	2,056		
Total	298,432	19			

Comparative analysis of transgenic plant production rates across the four developmental stages (V_1- V_4) revealed significant differences.

The highest percentage of transgenic plants was achieved during the V_2 stage, which corresponds to a capitulum size of 2-2.2 cm. This stage proved to be the most optimal for genetic transformation. The V_1 stage, with a capitulum size of 1.6-1.9 cm, and the V_3 stage, with a capitulum size of 2.5-2.7 cm, also demonstrated considerable success rates but lower rate the stage V_2. These findings underscore the critical importance of selecting the appropriate developmental stage of the capitulum to maximize the efficiency of genetic transformation (figure 2).

On the othere hand, a significantly lower rate of transgenic plants was observed during period V_4 (2.8-3.0 cm), suggesting that the optimal developmental stage for achieving high transgenic plant rate is well-defined. During perioade V_4 the efficiency of transgenic plant production declines markedly. Furthermore, polynomial analysis demonstrated a strong correlation between capitulum size and the percentage of transgenic plants obtained, highlighting the critical influence of developmental stage on transformation efficiency (figure 3).

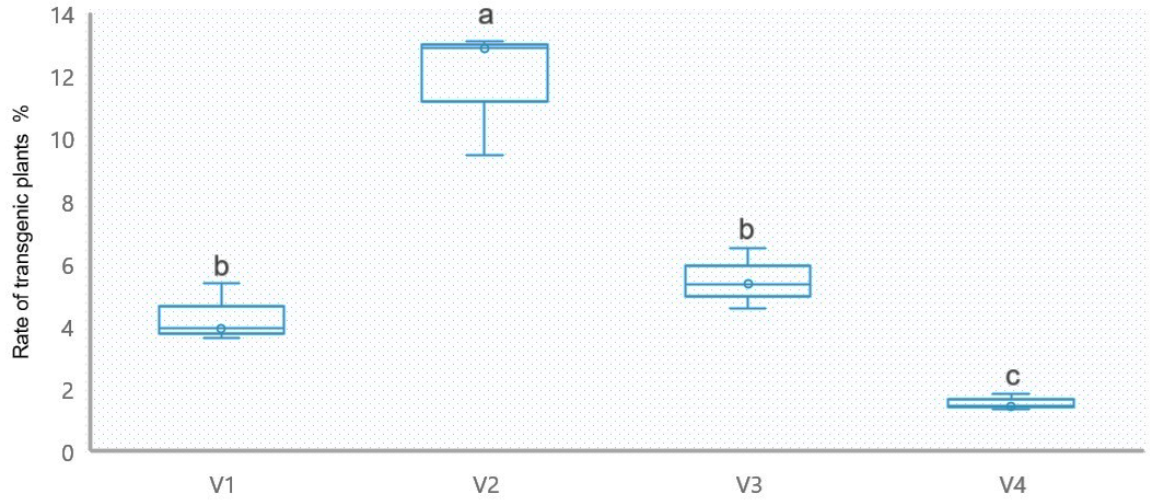

Figure 2. Boxplot representation of the procentage of transgenic plants obtaind according to the stage of develoopment for sunflowt bud: (V_1)- 1.6-1.9 cm, (V_2)- 2.0-2.2, (V_3) - 2.5-2.7 cm and (V_4) - 2.8-3.0 cm.

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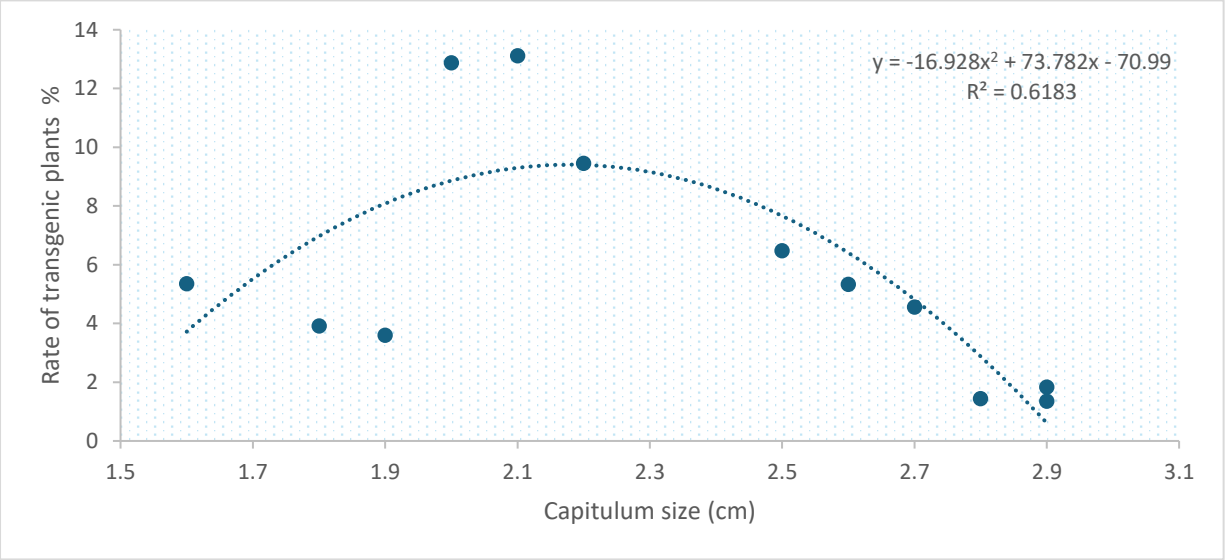

Figure 3. Polynomial analysis of the relationship between capitonnage and yield rate of transgenic sunflower plants

The results suggest that the developmental stage of the capitulum has a significant impact on genetic transformation efficiency. An optimal capitulum size maximizes the rate of obtaining transgenic plants (figure 3).

Studies by Birch (1997) [34] highlight the challenges encountered in the practical application of genetic transformation systems and propose strategies to enhance efficiency and facilitate large-scale implementation.

Therfore, these studies emphasize that the methodology for obtaining transgenic plants is both applicable and useful on a large scale. However, there are several limitations that could influence the rate of resulting transgenic plants [35].

The study focused on a single inbred line, HA-291, and the findings may not be universally applicable to all sunflower varieties [36], [37]. Additionally, it is important to note that head size can be influenced by environmental conditions, meaning it does not necessarily dictate the optimal stage for obtaining transgenic plants.

In future research, it is essential that the results focus more on the number of days from bud appearance to flowering, to accurately reflect the developmental stage of sunflowers [38]. Moreover, regarding the *Agrobacterium* strains used, different strains and vectors could offer varying transformation efficiencies. Employing alternative strains could potentially improve growth rates [39].

The use of GUS assays to confirm transformation might not fully represent the efficiency of genetic insertion. Other confirmation methods, such as molecular techniques (e.g., PCR) [40], [41] could provide more precise results. In the context of CRISPR/Cas9 technology [42], the focus remains primarily on *Agrobacterium*-mediated transformation [43]. A comparative analysis involving CRISPR/Cas9 could offer insights into the relative efficiency and benefits of each method.

Optimizing the biotechnological transformation method for sunflowers could bring significant benefits in combating diseases and pests, as well as improving the nutritional quality of the crops.

Conclusions

In the present study, transgenic sunflower genotypes were created using the *Agrobacterium* strains EHA105 and LBA440. The results showed a strong correlation between the developmental stage of the capitulum and the rate of transgenic plant production, significantly influencing the percentage of obtained transgenic genotypes. This underscores the importance of selecting the correct developmental stage for maximum efficiency in genetic transformation.

In future research, other *Agrobacterium* strains and vectors will be considered, along with additional verification methods and a larger number of genotypes to further refine the transformation method.

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References

[1] Lomascolo, A., Uzan-Boukhris, E., Sigoillot, J.-C., & Fine, F. (2012). *Rapeseed and sunflower meal: a review on biotechnology status and challenges*. Applied Microbiology and Biotechnology, 95(5), 1105–1114.

[2] Emilian, O., Ioan, S., Irina, P., Raul, P., Adriana, C., Dorin, C., & Ciprian, S. (2023). *Cytological Applications of the Vacuolization Phenomenon as a Means of Determining Saline Cytotoxicity*. Applied Sciences, 13(14), 8461.

[3] Popescu, A., Dinu, T.A., Stoian, E., & Şerban, V. (2023). *Climate change and its impact on wheat, maize and sunflower yield in Romania in the period 2017-2021*. Scientific Papers Series Management, Economic Engineering in Agriculture & Rural Development, 23(1).

[4] Rud, V. P., Mohylna, O. M., Terokhina, L. A., & Iljinova, Y. M. (2023). *Global overview of the onion market and production prospects in Ukraine*. Vegetable and Melon Growing, (73), 97-105.

[5] El-Hamidi, M., & Zaher, F. A. (2018). *Production of vegetable oils in the world and in Egypt: an overview*. Bulletin of the National Research Centre, 42(1), 19.

[6] González, F.G., Capella, M., Ribichich, K.F., Curín, F., Giacomelli, J.I., Ayala, F., Watson, G., Otegui, M.E., & Chan, R.L. (2019). *Field-grown transgenic wheat expressing the sunflower gene HaHB4 significantly outyields the wild type*. Journal of Experimental Botany, 70(5), 1669-1681.

[7] Onisan, E., Sarac, I., Petrescu, I., & Dorin, C. (2023). *New breeding methods in sunflower hybrid research and development*. Scientific Papers. Series A. Agronomy, 66(1).

[8] Radanović, A., Cvejić, S., Jocković, M., Dedić, B., Jocić, S., & Miladinović, D. (2023). *Conventional and molecular breeding for sunflower nutrition quality improvement*. In A. Raina, M. R. Wani, R. A. Laskar, N. Tomlekova, & S. Khan (Eds.), Advanced Crop Improvement, Volume 2: Case Studies of Economically Important Crops (pp. 351–391).

[9] Onișan, E., Botău, D., Petrescu, I., & Sărac, I. (2023). *In vitro and in vivo embryo rescue involvement in the economy of sunflower breeding*. Journal of Horticulture, Forestry and Biotechnology, 27(1), 12-19.

[10] Bonciu, E., & Sarac, I. (2017). *Implications of modern biotechnology in the food security and food safety*. Annals of the University of Craiova-Agriculture, Montanology, Cadastre Series, 46(1), 36-41.

[11] Dezar, C. A., Gago, G. M., González, D. H., & Chan, R. L. (2005). *Hahb-4, a sunflower homeobox-leucine zipper gene, is a developmental regulator and confers drought tolerance to Arabidopsis thaliana plants*. Transgenic Research, 14(4), 429–440.

[12] Gupta, S. K. (2015). *Breeding Oilseed Crops for Sustainable Production: Opportunities and Constraints*. Academic Press.

[13] Onisan, E., Petrescu, I., & Sarac, I. (2020*). The difficulty in obtaining Orobanche cumana sunflower resistance hybrids*. Annals of the University of Craiova-Agriculture, Montanology, Cadastre Series, 49(2), 125-130.

[14] Onișan, E., Sărac, I., Dotău, D., & Petrescu, I. (2021). *Effects of metalaxyl on plant development and germination capacity of sunflower hybrids*. Journal of Horticulture, Forestry and Biotechnology, 25(4), 82-85.

[15] Pérez-de-Luque, A., Fondevilla, S., Pérez-Vich, B., Aly, R., Thoiron, S., Simier, P., Castillejo, M.A., Fernández-Martínez, J.M., Jorrín, J., Rubiales, D., & Delavault, P. (2009). *Understanding Orobanche and Phelipanche–host plant interactions and developing resistance*. Weed Research, 49, 8-22.

[16] Cuccurullo, A., Nicolia, A., & Cardi, T. (2022). *Resistance against broomrapes (Orobanche and Phelipanche spp.) in vegetables: a comprehensive view on classical and innovative breeding efforts*. Euphytica, 218(6), 82.

[17] Frey, M., Vahabi, K., Cankar, K., Lackus, N.D., Padilla-Gonzalez, F., Ro, D.K., Rieseberg, L., Spring, O., & Tissier, A. (2024). *Sesquiterpene Lactones–Insights into Biosynthesis, Regulation and Signalling Roles*. Critical Reviews in Plant Sciences, 1-27.

[18] Sisou, D., Tadmor, Y., Plakhine, D., Ziadna, H., Hübner, S., & Eizenberg, H. (2021). *Biological and transcriptomic characterization of pre-haustorial resistance to sunflower broomrape (Orobanche cumana W.) in sunflowers (Helianthus annuus)*. Plants, 10(9), 1810.

[19] Cantamutto, M., & Poverene, M. (2010). *Transgenic sunflower*. In Genetics, Genomics and Breeding of Sunflower (pp. 279-312).

[20] Bidney, D. L., & Scelonge, C. J. (1997). *Sunflower Biotechnology*. In Sunflower Technology and Production (pp. 559–593). John Wiley & Sons, Ltd.
[21] Sala, C.A., Bulos, M., Altieri, E., & Ramos, M.L. (2012). *Genetics and breeding of herbicide tolerance in sunflower*. Helia, 35(57), 57-70.
[22] Sheri, V., Muddanuru, T., & Mulpuri, S. (2021). *Genetic engineering of sunflower (Helianthus annuus L.) for important agronomic traits*. In P. B. Kavi Kishor, M. V. Rajam, & T. Pullaiah (Eds.), Genetically Modified Crops: Current Status, Prospects and Challenges Volume 1 (pp. 175–200)
[23] Sujatha, M., Vijay, S., Vasavi, S., Reddy, P. V., & Rao, S. C. (2012). *Agrobacterium-mediated transformation of cotyledons of mature seeds of multiple genotypes of sunflower (Helianthus annuus L.)*. Plant Cell Tissue and Organ Culture, 110(2), 275–287.
[24] Rauf, S. (2019). *Breeding strategies for sunflower (Helianthus annuus L.) genetic improvement*. In J. M. Al-Khayri, S. M. Jain, & D. V. Johnson (Eds.), Advances in Plant Breeding Strategies: Industrial and Food Crops (pp. 637-673).
[25] Pons, E., Peris, J. E., & Peña, L. (2012). *Field performance of transgenic citrus trees: Assessment of the long-term expression of uidA and nptII transgenes and its impact on relevant agronomic and phenotypic characteristics*. BMC Biotechnology, 12(1), 41.
[26] Mabuza, L. M., Mchunu, N. P., Crampton, B. G., & Swanevelder, D. Z. H. (2023). *Accelerated breeding for Helianthus annuus (Sunflower) through doubled haploidy: An insight on past and future prospects in the era of genome editing*. Plants, 12(3), Art. no. 3.
[27] Bisht, D. S., Bhatia, V., & Bhattacharya, R. (2019). *Improving plant-resistance to insect-pests and pathogens: The new opportunities through targeted genome editing*. Seminars in Cell & Developmental Biology, 96, 65-76. Academic Press.
[28] Singh, R. K., Prasad, A., Muthamilarasan, M., Parida, S. K., & Prasad, M. (2020). *Breeding and biotechnological interventions for trait improvement: status and prospects*. Planta, 252, 1-18.
[29] Mehaboob, V. M., et al. (2022). *Somatic Embryogenesis and Transformation Studies in Ginger*. In Biotechnology and Crop Improvement. CRC Press.
[30] Rodrigues, S. D., et al. (2021). *Efficient CRISPR-mediated base editing in Agrobacterium spp*. Proceedings of the National Academy of Sciences, 118(2), e2013338118.
[31] Lardet, L., et al. (2011). *Variation in GUS activity in vegetatively propagated Hevea brasiliensis transgenic plants*. Plant Cell Reports, 30(10), 1847–1856.
[32] Birch, R. G. (1997). *Plant transformation: problems and strategies for practical application*. Annual Review of Plant Biology, 48(1), 297-326.
[33] Andow, D. A., & Zwahlen, C. (2006). *Assessing environmental risks of transgenic plants*. Ecology Letters, 9(2), 196-214.
[34] Göksoy, A. T., Demir, A. O., Turan, Z. M., & Dağüstü, N. (2004). *Responses of sunflower (Helianthus annuus L.) to full and limited irrigation at different growth stages*. Field Crops Research, 87(2-3), 167-178.
[35] Chapman, S. C., Hammer, G. L., & Meinke, H. (1993). *A sunflower simulation model: I. Model development*. Agronomy Journal, 85(3), 725-735.
[36] Villalobos, F. J., Hall, A. J., Ritchie, J. T., & Orgaz, F. (1996). *OILCROP-SUN: A development, growth, and yield model of the sunflower crop*. Agronomy Journal, 88(3), 403-415.
[37] Brasileiro, A. C. M., Leplé, J. C., Muzzin, J., Ounnoughi, D., Michel, M. F., & Jouanin, L. (1991). *An alternative approach for gene transfer in trees using wild-type Agrobacterium strains*. Plant Molecular Biology, 17, 441-452.
[38] Wassenegger, M. (2001). *Advantages and disadvantages of using PCR techniques to characterize transgenic plants*. Molecular Biotechnology, 17(1), 73-82.
[39] Holst-Jensen, A., Rønning, S. B., Løvseth, A., & Berdal, K. G. (2003). *PCR technology for screening and quantification of genetically modified organisms (GMOs)*. Analytical and Bioanalytical Chemistry, 375, 985-993.
[40] Mei, Y., Wang, Y., Chen, H., Sun, Z. S., & Ju, X.-D. (2016). *Recent progress in CRISPR/Cas9 technology*. Journal of Genetics and Genomics, 43(2), 63-75.
[41] Guo, M., Bian, X., Wu, X., & Wu, M. (2011). *Agrobacterium-mediated genetic transformation: history and progress*. In Genetic Transformation. IntechOpen.
[42] Y. Mei, Y. Wang, H. Chen, Z. S. Sun, & X.-D. Ju. (2016). *Recent progress in CRISPR/Cas9 technology*. Journal of Genetics and Genomics, 43(2), 63-75.