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REVIEW ARTICLE

Recent efforts to increase Soybean (*Glycine max* L.) tolerance to abiotic stresses using CRISPR-Cas technology

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Abstract

In many countries, soybeans are an important cash crop because of their application in food, feed and biodiesel production. However, traditional breeding methods have limitations in producing the huge yields required by agriculture. Furthermore, soybean germplasm genetic diversity has decreased over time as a result of domestication and selective breeding. In this regard, novel molecular breeding technologies, including genome editing, have been developed to precisely modify agronomically desired characteristics such as yield, quality and abiotic/biotic stress tolerance. Biotic and abiotic stresses and the demand for higher crop yields and nutritional value are providing new difficulties to modern agriculture. Precision breeding utilizing CRISPR-Cas9 technology emerged as one of the most effective methods in crop production. Although many scientists are working on increasing soybean tolerance against different abiotic stresses, new modern approaches are still needed. This review discusses recent efforts devoted to solving salinity, drought and heat tolerance, flood resistance, disease resilience and crop improvement problems that utilized CRISPR-Cas9 technology. Furthermore, novel tools, such as virus-induced genome editing (VIGE) and omics-based approaches, integrated with the CRISPR/Cas9 platform, will be discussed. The opportunities and limitations of incorporating CRISPR technology into global agricultural systems are thoroughly examined. In total, we reviewed 54 papers from the NCBI database and the ScienceDirect portal. The outcomes highlighted in this work show the significance of CRISPR-Cas9 technology in improving soybean tolerance to abiotic stresses.

Keywords: CRISPR-Cas9; Soybean (*Glycine max* L.); TALEN; VIGE; ZFN

Introduction

Climate change continually undermines food security and the system of farming. The agriculture sector faces significant obstacles in meeting sustainable development goals as a result of the ongoing consequences of climate change (1). Climate change has caused severe storms and droughts, threatening several countries' water supplies and food sustainability. In addition, sea-level rise causes coastal flooding and erosion, endangering populations and infrastructure in low-lying areas (2). Flooding is the second major cause of abiotic stress, inflicting financial damages of around $1.5 billion/yr in the USA's farming (3). Flooding stress adversely impacts soybean growth and yield. Molecular tools such as QTL mapping and CRISPR-Cas9 have identified key genomic regions governing flood tolerance. Advances in transcriptome analysis and genome-wide association studies (GWAS) complement the development of flood-resistant cultivars (4).

As a result of their adaptation to shifting climatic circumstances, bacteria, fungi, insects and pests have become more problematic for the agricultural sector (5-7). Plants are often exposed to various stresses, which have permanent consequences for agricultural productivity (8). Abiotic stresses are one of the most serious risks to many crops, as well as soybean (*Glycine max* L.) and their growth and yields worldwide (9). CRISPR/Cas9 is a third-generation gene editing technique that has quickly become one of the most effective, easy, affordable and user-friendly ways for targeted gene editing (10). In this manner, CRISPR-Cas9 technology can be efficiently used to improve plant stress resistance and crop yield, which may serve both food security and sustainable agriculture (11).

Soybean is one of the crops that was approached by using CRISPR-Cas9 to develop new lines with higher stress resistance (12). Soybeans are sensitive to heat and drought stress, which affects production by restricting physiological development. Stress-induced yield loss is more common when soybeans receive limited fertilizer inputs (13). However, boosting agronomically favourable soybean characteristics under drought, salt and heat conditions can be challenging since there is trait complexity and a multitude of variables influencing production. Genome editing in soybeans started with ZFN and TALEN technologies, but CRISPR-Cas developed and quickly established the preferred strategy for soybean genome modification due to its greater precision, ease of use and cost-effectiveness (14).

CRISPR is a critical tool for making groundbreaking advances in plant genomic development (15). The CRISPR-Cas9 system, derived from bacterial immune mechanisms, facilitates targeted genome editing by inducing site-specific mutations. This revolutionary technology has significantly advanced reverse genetics and functional genomics, enabling precise gene manipulation to enhance stress tolerance and crop quality (16). Scientists identified many CRISPRs, including Cas9, Cpf1 and Cas12a, that can enhance certain gene expression in plants (17). With the improvement of precise gene editing tools, it is now possible to create plants with desirable characteristics that include increased yields, resistance to diseases and nutritional composition (18). This advanced genome-editing technology focuses on features that increase crop cost-efficacy in the face of both abiotic and biotic stresses. To date, CRISPR-Cas9 has been applied to several crop species to improve various characteristics, including yield optimization and resistance to environmental and pathogenic challenges. Over the past five years, genome-editing research has predominantly focused on major crops, targeting genes associated with improving male sterility, resistance against pathogens and nutritional value (16).

This review highlights CRISPR-based advances in addressing abiotic stresses like salinity, flooding and heat alongside biotic stress management and nutritional improvements in soybeans. In previous studies, we extensively reviewed maize (*Zea mays*) and cotton tolerance mechanisms under the aforementioned abiotic stresses in our published works (19-21).

Increasing salinity tolerance in Soybean using CRISPR/Cas9 technology

Alongside conventional breeding methods, CRISPR/Cas9 is employed to develop salt-tolerant soybean cultivars, ensuring sustainable yields under dynamic environmental conditions while preserving genetic diversity for future applications (9). Salinity affects a significant proportion of arable land, posing challenges to crop productivity. CRISPR/Cas9 technology has been effectively employed to enhance salinity tolerance in soybeans. A notable example is the significant DNA segment deletion in lncRNA77580, which affects nearby gene expression related to salt stress response (22).

Plant development is linked with various aspects, including the stress factors that affect the expression of genes to be regulated by remodelling chromatin (23). Genes involved in adaptation to various stressors have been identified in many key model plants. Transgenic approaches, such as overexpressing or silencing these genes, provide valuable insights into their roles in stress tolerance. Since stress tolerance is typically a quantitative trait, it is developed by the contribution of many genes that provide substantial levels of resistance (24).

Sucrose non-fermenting-related kinases (SnRKs) are pivotal in regulating plant growth. They are also involved in stress responses; GmSnRK1.1 and GmSnRK1.2 are required for stress resistance in soybeans (*Glycine max*) resulting from ABA and alkaline conditions. In one work, GmSnRK1.1/GmSnRK1.2 double-knockout (DKO) hairy roots were obtained by using a CRISPR/Cas9-based dual-gRNA system. Phenotypic analyses revealed that under 25 μM ABA, DKO roots exhibited reduced growth inhibition compared to wild-type and overexpression lines, whereas under 50 mM $NaHCO_3$, DKO roots showed heightened sensitivity. These findings show the differential roles of these two genes in modulating ABA susceptibility and alkaline stress tolerance (25). GsSnRK1.1 was responsive to stress resulting from saline-alkali and drought in wild soybeans. A response was also observed to ABA stress. Mutant analyses revealed that kinase activity is essential for stress tolerance, as kinase-dead variants were ineffective. Complementation studies in yeast and transgenic *Arabidopsis* verified the involvement of GsSnRK1.1 in maintaining cellular homeostasis during abiotic stress conditions (26). The overexpression of GmNHL1 in soybeans improved salt tolerance by maintaining root integrity, reducing oxidative damage and enhancing antioxidant enzyme activity. Knockout lines demonstrated reduced tolerance, suggesting GmNHL1 is a key target for improving salt stress adaptation (27). GmMYB118, a nuclear-localized MYB transcription factor, was found to regulate stress-associated genes and maintain osmotic balance under drought and salt stresses. Overexpressed lines showed improved tolerance through higher proline and chlorophyll levels, while CRISPR-mediated knockouts exhibited reduced tolerance. These findings position GmMYB118 as a critical regulator of soybean abiotic stress responses (28). A NAC transcription factor (GmNAC06) was identified as a regulator of salt stress tolerance in soybeans. Overexpression of GmNAC06 increased proline and glycine betaine levels and maintained Na^+/K^+ homeostasis, mitigating oxidative stress. In contrast, knockout mutants exhibited reduced tolerance. Functional analyses indicated that GmNAC06 influences both hairy root systems and whole-plant phenotypes, highlighting its utility in developing salt-tolerant soybean cultivars (29).

MicroRNAs (miRNAs) play a crucial role in regulating the levels and distribution of target mRNAs over time and space, thereby influencing various plant processes. They are involved in almost every stage of plant development, including the shift from juvenile to adult, the growth of the shoot's apical meristem, the morphogenesis of leaves, the production of floral organs and the timing of flowering (30). They are generated from DNA and participate in the regulation of other genes involved in protein synthesis; they regulate other protein-coding genes (31). The transcription of miRNA-encoding genes (MIR genes) is controlled by a range of transcription factors, many of which are themselves influenced by other miRNAs. Modulating the activity of MIR genes or miRNAs appeared as an effective biotechnological approach to managing plant metabolism against biotic and abiotic stresses. Current strategies for miRNA regulation involve either increasing or decreasing MIR gene activity, alongside employing genetic engineering tools to adjust miRNA concentrations within cells. Techniques such as transgenesis, cisgenesis, intragenesis, synthetic MIR genes, target mimicry (both natural and artificial) and CRISPR-based methods (e.g., CRISPR/Cas9, Cpf1, dCas9 and CRISPR13a) have been utilized for miRNA modulation. Topical delivery systems and epigenetic memory mechanisms have also been investigated. While these advanced biotechnological tools have demonstrated potential for improving traits in both model and crop plants, their respective advantages and limitations remain to be fully understood. One work outlines the functional roles of miRNAs in stress responses, critically examines the new biotechnological tools (NBTs) used to manipulate MIR genes and miRNAs and highlights recent findings in this field. Additionally, it evaluates the benefits and challenges of these tools and proposes solutions to address existing gaps. Finally, future research directions and opportunities for enhancing miRNA modulation in economically important crops are discussed (32). Research summarizes numerous study efforts, including advances in miRNA -mediated gene regulation and its role in plant development, to lay the groundwork for further in-depth investigation into miRNA function and the discovery of the molecular mechanisms that underlie miRNA-pathway interactions (30).

The combination of CRISPR/Cas9 technology and traditional breeding methods has considerably accelerated the production of salt-tolerant soybean cultivars, ensuring consistent yields in severe environmental circumstances. Researchers discovered several molecular processes that improve tolerance to salinity, drought and alkaline stress by targeting critical regulatory genes, transcription factors, kinases and microRNAs involved in stress responses (Table 1).

Development of various methodologies to enhance drought and heat stress tolerance in Soybean

Although soybeans (*Glycine max*) are key oilseed crops farmed worldwide, abiotic stresses, particularly drought, significantly impact their yield. Identifying and breeding drought-tolerant soybean varieties should be prioritised. After all, there is a clear need to increase agricultural production in the face of limited water supplies and rising food demand. However, due to the difficulty in phenotyping and genotyping, breeding for drought tolerance is typically overlooked (33). Research indicates that GmNF-YC14, a crucial gene in soybeans that is essential for the plant's capacity to withstand drought and salt stress. The gene functions as part of an ensemble (a heterotrimer with GmNF-YA16 and GmNF-YB2) to activate an abscisic acid (ABA) signalling pathway through a different gene, GmPYR1. When the GmNF-YC14 gene was removed using CRISPR/Cas9, the mutant plants became more vulnerable to drought. In contrast, overexpressing GmNF-YC14 or GmPYR1 in field experiments resulted in higher yield, fuller grains and thicker stems during dry circumstances. This study discloses a new function for NF-Y transcription factors, implying that GmNF-YC14 and GmPYR1 are attractive genetic targets for enhancing soybean drought tolerance (34). The investigators looked into how a specific microRNA (gma-miR398c) influences drought tolerance in soybeans. Using a multi-omics approach, the researchers discovered that miRNA functions as a negative regulator of drought resistance by repressing a collection of genes (GmCSD1a/b, GmCSD2a/b/c and GmCCS) implicated in the peroxisome pathway, which is important for oxidative stress management. Drought induces gma-miR398c, which then represses genes involved in the scavenging of damaging superoxide radicals. This inhibition causes increased oxidative damage, as seen by higher electrolyte leakage and bigger stomatal openings, making the plant more susceptible to water loss. Overexpressing gma-miR398c lowered drought tolerance (reduced germination, increased water loss), but taking it out increased tolerance. An alternate splicing process in the target genes was also discovered, which could allow the plant to circumvent this miRNA's regulation. Finally, the investigation identified gma-miR398c as a critical negative regulator that increases soybean sensitivity to drought by affecting the plant's antioxidant defense mechanism (35).

In another study, GmNAC8, a nucleus-localized transcription factor, was upregulated under drought, ABA, ETH and SA treatments. Overexpression of this gene was found to increase drought tolerance, which was in correlation with enhanced levels of proline and SOD, while knockout lines showed lower tolerance. Interaction studies determined the physical association of GmNAC8 with GmDi19-3, suggesting a collaborative regulatory mechanism.

Table 1. Comparison of conventional breeding and CRISPR-Cas technology

Aspect	Conventional breeding	CRISPR-Cas genome editing
Accuracy	Depends on spontaneous genetic recombination; alterations are indirect and less predictable.	Extremely precise; enables targeted alteration of certain genes with few off-target consequences.
Frequency of development	Time-consuming (it may take multiple generations to attain desired features).	Enhanced soybean cultivars develop more quickly and in fewer generations.
Genetic diversity	Repeated selection of similar traits results in diminished genetic diversity over time.	Preserves or creates new varieties by directly altering or introducing beneficial alleles.
Stress tolerance	Limited success in increasing resistance to abiotic conditions such as drought, salinity, heat and flooding.	Direct editing of important stress-related genes (e.g., GmNHL1, GmMYB118, GmNAC06) effectively improves tolerance.
Trait complex management	It is difficult to address polygenic features such as drought and salinity tolerance.	Provides for the simultaneous manipulation of many genes (for example, utilizing dual gRNA systems) in order to handle complicated features.
Gene identification and verification	Relying on phenotypic selection makes it hard to swiftly verify the functions of genes.	Combines omics-based techniques with functional investigations to quickly validate gene roles and stress circuits.
Application examples	Cross-breeding to improve yield, quality and stress tolerance.	Used to improve drought (GmNF-YC14, GmNAC8, GmHsps_p23-like), salt (GmNHL1, GmMYB118) and heat tolerance (GmHsp90A2).
Incorporation with Omics	Low interaction with molecular data.	Integrates transcriptomics, proteomics and metabolomics to improve gene targeting and comprehension.
Cost-effectiveness	Long breeding cycles result in substantial long-term costs, despite relatively cheap beginning expenditures.	Cost-effective over time due to faster outcomes and shorter breeding cycles.
Future potential	There are limited options for addressing the effects of climate change on soybeans.	When combined with omics, VIGE and conventional breeding, it has great potential for climate-resilient crops.

These results establish GmNAC8 as a regulator of drought tolerance in soybeans (36). GmNAC12, a nuclear-localized NAC transcription factor, was upregulated during drought and hormonal treatments. The plant lines overexpressing the gene showed significantly higher survival rates under drought conditions, while knockout lines displayed reduced tolerance. Proteomic analysis identified 185 interaction partners, implicating GmNAC12 in diverse stress-related pathways. These findings suggest that GmNAC12 is a key regulator of soybean drought tolerance (37). Overexpression of GmNF-YC14 enhanced drought tolerance and agronomic traits, while knockout lines were more sensitive. These results highlight the potential of the NF-Y-PYR module in improving soybean stress resilience (34).

Research indicates that the GmHsps_p23-like gene in soybean (*G. max*) is connected with drought tolerance. RT-PCR was used to clone the gene, followed by bioinformatics analysis and the creation of overexpression and gene-editing vectors. Transgenic soybeans were developed using Agrobacterium-mediated transformation and evaluated using PCR, Southern blotting and drought stress tests. Overexpression of GmHsps_p23-like resulted in greater drought tolerance, as demonstrated by higher SOD, POD and PRO levels and decreased MDA content under drought circumstances. In contrast, altered plants displayed lower drought resilience. It was the first report of cloning and functional testing of the GmHsps_p23-like gene, laying the groundwork for the development of drought-resistant transgenic soybean cultivars (38). GmHsp90A2 is another vital gene that was found to positively regulate heat tolerance in soybean. Transgenic lines overexpressing GmHsp90A2 exhibited enhanced chlorophyll retention and reduced malondialdehyde (MDA) levels under heat stress, whereas knockout lines demonstrated the opposite phenotype. Protein interaction assays indicated that GmHsp90A2 forms a complex with GmHsp90A1, which translocated to the nucleus, reinforcing its role in thermotolerance (39). Research indicates that molecular and traditional techniques to improve soybean heat stress resistance. Although techniques like as QTL mapping, genetic engineering, transcription factors, transcriptome analysis and CRISPR have enhanced tolerance, the full genetic pathway remains unknown. Traditional breeding and hormonal treatments are also useful. Future advancement is dependent on the combined application of these technologies and additional research to find genetic elements governing heat stress responses, which will aid in the development of heat-tolerant soybean cultivars (40). The study highlighted the recent developments in phytohormones and their corresponding microorganisms in *Glycine max* thermotolerance by integrating plant-microbial interactions. They proposed that beneficial microorganisms under heat stress (HS) can produce thermotolerance and thermomorphogenesis via a variety of complex pathways. For the first time, researchers have gained insight into the microbial-mediated phytohormone signalling route for transcriptional control of secondary metabolism in diverse soybean HS tolerances. According to the authors' conclusion, a primary perspective on enhancing soybean plant response to HS and creating important phytohormones through microbial-mediated and secondary metabolite interactions has been described (41).

Recent developments in molecular biology, genetic engineering and multi-omics methods have greatly improved our understanding of drought and heat stress tolerance in soybean. Several essential regulatory genes have been identified as important contributors to stress resilience due to their involvement in ABA signalling, oxidative stress control and protein stability under adverse conditions. In contrast, miRNAs have been identified as a negative regulator of drought tolerance by decreasing antioxidant defense genes, demonstrating the intricacy of regulatory networks. Overexpression experiments with CRISPR/Cas9 gene editing, Agrobacterium-mediated transformation and transcriptome profiling have shown increased drought and heat tolerance, improved agronomic features and superior physiological performance under stress. Furthermore, current research highlights the importance of beneficial plant-microbe interactions in modifying phytohormone signalling pathways and promoting thermotolerance.

Virus-Induced Genome Editing (VIGE) to increase stress tolerance in Soybean

Functional omics, including transcriptomics, proteomics and metabolomics, have deepened our understanding of stress-responsive genes. These insights, coupled with CRISPR-based validation, facilitate the development of stress-tolerant crops (42, 43). Viral delivery is one of the methods of CRISPR/Cas9 constructs deserving attention, as it offers improved gene editing efficiency and reduces regulatory burdens. However, points such as virus host range, tissue specificity, cargo capacity, transmission mode and biosafety must be carefully had to be taken into account while developing viral vectors for this purpose (44). The soybean GmGlb1-1 gene, associated with nitric oxide turnover, was overexpressed to confer resistance to *Meloidogyne incognita*. Transgenic lines exhibited reduced nematode susceptibility and upregulated defence-related genes, enhancing oxidative stress responses and improving systemic acclimation to biotic and abiotic stresses (45). The MADS-box transcription factor PsMAD1 plays a significant role in zoosporogenesis and the pathogenicity of *Phytophthora sojae*. As key regulators, MADS-box transcription factors are conserved across eukaryotes and influence various biological processes, including pathogen development and virulence. In *P. sojae,* the PsMAD1 gene was identified as the sole MADS-box gene and exhibited high expression levels in the sporangia formation and infection period. In one work, the CRISPR/Cas9 system was used to create PsMAD1 knockout mutants to explore its function. The mutants displayed normal vegetative growth, which corresponded with oospore production and tolerance to abiotic stresses. The plants failed to release zoospores due to impaired cytoplasmic cleavage into uninucleate zoospores, as revealed by microscopy. Additionally, the mutants showed lower pathogenicity in soybeans. Transcriptomic analysis further suggested that PsMAD1 regulates numerous genes associated with zoospore development and infection processes. These findings position PsMAD1 as a pivotal regulator in the lifecycle and pathogen resistance of *P. sojae* (46).

The combination of VIGE and CRISPR/Cas9 technology is a viable strategy for improving soybean stress tolerance and disease resistance. Using functional omics to discover stress-responsive genes, viral delivery techniques improve gene-editing efficiency while minimizing regulatory limitations. Successful applications, including overexpression and CRISPR/Cas9-mediated knockout, indicate the method's potential. However, viral vector design difficulties such as host range, cargo capacity and biosafety must be carefully addressed in order to maximize VIGE-based techniques for long-term soybean development.

Important genes involvement in plant stress

The efficacies of CRISPR/Cas9 in developing new varieties of soybeans have been shown in several works (47). PYL genes are one of the genes involved in plant stress tolerance. An early study established the involvement of *PYL* genes in drought stress tolerance in *Arabidopsis* (40). In soybeans, deletions of three genes, *GmPYL17*, *GmPYL18* and *GmPYL19*, responsible for abscisic acid (ABA) receptors, led to obtaining plants having less susceptibility to ABA compared to wild ones. Thus, the authors obtained three homozygous plants. Significant germination rate and root length changes were observed in double mutants compared to wild plants. The mutants showed no sensitivity to ABA treatment. Besides, significant differences were observed in the number of branches (48).

miR156b-GmSPL2b module is known to regulate male fertility in soybean plants under high-temperature stress. The overexpression of *miR156b* damages the metabolism of flavonoids and the clearance of ROS. *GmSPL2b* is considered involved in cytoplasmic male sterility (CMS). Its degradation was attributed to downstream carbon metabolism and sugar transportation, which interfered with ROS scavenging, leading to abnormal male fertility in CMS-based soybean lines. Thus, its manipulation was suggested as one of the sites to obtain heat-tolerant soybean plants (49). PDCT, Phosphatidylcholine: diacylglycerol choline phosphotransferase, is another protein involved in carbon metabolism (50). Knocking out *PDCT* genes in *Glycine max* (*GmPDCT*) using the CRISPR-Cas9 system in a soybean plant was suggested as an effective approach to obtaining seeds with higher levels of unsaturated oils. The downregulations of two isoforms, *GmPDCT1 and GmPDCT2,* were proven by establishing fatty acid composition in soybean seed oil (51). Multiple roles of unsaturated fatty acids in plant stresses of various origins (52) can link *GmPDCT* downregulation with plant stress. In one work, the dysfunction of *GmVPS8a*, a vacuolar protein sorting protein, was found to affect the metabolism of lipids and sugars. Significantly smaller plant height was observed in the mutated plants compared to the wild types. The petiole length was reduced by more than twice in engineered plants. Besides, significant reductions were established in the oil content and the content of C16:0 saturated and C18:2 double unsaturated fatty acids. Combined transcriptomic and proteomic analysis suggested that knocking out *GmVPS8a* affects auxin signal transduction and metabolism of sugar and lipids (53). The above-discussed results achieved by knocking out some soybean genes using CRISPR-Cas9 prove its efficacy in developing new soybean lines resistant to various stress factors. CRISPR/Cas9 is a promising strategy to regulate frameworks and significant barriers in plant development. Advances in precise gene editing tools and integration with conventional breeding methods hold the key to future breakthroughs in crop improvement. Emphasis should also be placed on optimizing delivery methods and expanding applications in underutilized crops (54).

Conclusion

Climate change causes significant problems to agriculture worldwide, such as a lack of water, extreme weather events and other biotic stresses linked with climate change. Soybean, a major oilseed crop, is especially susceptible to these stressors, resulting in severe output losses. Advances in molecular biology, notably CRISPR/Cas9 genome editing technology, have transformed our capacity to create stress-tolerant and high-yield cultivars. In this review, we emphasized the significance of the CRISPR/Cas9 method in dealing with abiotic stressors such as salt, floods, drought and heat and in improving biotic stress resistance and nutritional quality. Despite the progress in gene engineering, researchers face resolving issues like the complexities of stress-response features and regulatory barriers. Combining CRISPR-based technologies with conventional breeding and functional genomics is the key to long-term crop development. Future work should prioritize increasing applications to underused crops and improving delivery mechanisms to optimize genetic breakthroughs. Agriculture can adapt to climate change and fulfil the rising global need for food security by utilizing cutting-edge genome editing methods.

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Authors' contributions

MSA drafted the manuscript, critically edited the paper and contributed a subsection. AMA co-drafted the manuscript and assisted in critical editing and subsection preparation. ANY acquired and analyzed the literature. NSO contributed to literature acquisition and analysis. AAM assisted in analyzing the reviewed literature. BOM participated in compiling and analyzing the literature. ZHB and ZTB contributed to literature acquisition, thoroughly revised the article and approved the final draft. LKK assisted in literature analysis. IYA carried out a thorough revision and final approval of the manuscript. All authors have read and approved the final version of the manuscript.

Compliance with ethical standards

Conflict of interest: The Authors do not have any conflict of interests to declare.

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