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Mitigating Salinity Stress In Chickpeas: A Review Of Genetic And Transgenic Strategies

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Abstract: Chickpea (*Cicer arietinum*) is an important crop, not only in India but also globally. It is an important source of protein, especially in vegetarian diets. Salinity has been a critical issue in agriculture and crops like chickpeas are highly susceptible to salt stress. Due to salinity, chickpeas experience high yield losses and many physiological disorders. Key findings from studies show that salinity stress significantly reduces seed germination, plant height, chlorophyll content, and overall plant vigor. Advancements in biotechnology, especially the application of genetic engineering and genomic technologies, have played a crucial role in the development of chickpea varieties that can tolerate salt. Incorporating the OsRuvB gene into chickpea using Agrobacterium-mediated transformation has been found to enhance resistance to salt stress. Furthermore, applying melatonin externally and utilizing plant growth-promoting microorganisms such as *Bacillus tequilensis* have demonstrated potential in alleviating the negative effects of salinity. These biotechnological approaches and agricultural methods show significant promise for improving chickpea yield in salty conditions, thereby contributing to global food security and sustainable farming practices.

Keywords - Chickpea (Cicer arietinum L.), salinity stress, genetic engineering, salt tolerance, OsRuvB gene, *Bacillus tequilensis*

I. INTRODUCTION

The chickpea, or Cicer arietinum L., is significant in history as it was one of the first grains crops that people farmed. It is currently ranked third in the world among food legumes, after field peas (Pisum sativum L.) and beans (Phaseolus spp.) (FAO 2008). Though more than 50 countries grow chickpeas, only 22 grow more than 20,000 hectares annually: 19 grow between 10,000 and 20,000 hectares. 8.4 million tonnes are produced worldwide each year; the top producers are Mexico (1.5%), Australia (1.5%), Pakistan (10%), Turkey (7%), Iran (3%), Myanmar (2%), India (65% of output), and Turkey (10%) (FAO 2008). Ethiopia, Iraq, Israel, Jordan, Morocco, and Syria are further producers; growing producers include Tanzania, Malawi, and Canada. Desi & kabuli are the two primary types of lentils. Whereas kabuli chickpea have larger seeds with having 'rams-head'-shaped seed coatings that are beige or cream in hue., Desi chickpeas are usually small-seeded with colored seed coverings and an acute angle. While there is a chance of hybridization between the two varieties, consumers and chefs have strong preferences for either kabuli or desi chickpeas. Chickpea, with their roughly twenty-one percent protein concentration, are a valuable source of protein for cereal-based diets. This is especially true in areas where the cost of animal protein is high or where vegetarianism is prevalent. Chickpea are a great low-input crop that fix up to 140 kg N ha-1 year-1 and provide over 70% of the nitrogen they need through symbiotic N2 fixation. This helps to improve soil fertility. Generally grown as cool-season crops, chickpeas are irrigated and rainfed (>90%), maturing frequently in the the year's warmest and driest months. Diseases like ascochyta blight (*Ascochyta rabiei*), fusarium wilt (*Fusarium oxysporum f. sp. ciceri*), and *botrytis grey mold* (Botrytis cinerea) are among the main obstacles to chickpea production, as are pests like the Helicoverpa pod borer (*Helicoverpa armigera* and *H. punctigera*) and leaf miner (*Liriomyza cicerina*) (Nene & Reddy 1987; Reed et al. 1987). Abiotic restrictions include soil salinity, freezing temperatures, and dryness.

II. Salinity Effects on Chickpea

Numerous studies under salinity stress have documented a decline in chickpea plant growth stages, seed output, and yield components (Sohrabi et al., 2008; Kandil et al., 2012; Pushpavalli et al., 2020). Worldwide, cultivated crops face serious abiotic stress from salinity in the soil. Only 150 x 107 hectares of the 700 x 107 hectares of arable land that are available for agriculture are appropriate for farming; the remaining 56 x 107 hectares are sodic and the remaining 34 x 107 hectares are saline. Furthermore, the area impacted by salt is always growing (Shahid et al., 2018). Every year, salinization, waterlogging, and sodification render almost 10 million hectares of irrigated land unusable (Szabolcs, 1989). The majority of these salt-affected areas are located in areas that are semi-arid or dry, where evapotranspiration rates are noticeably higher than in other regions and low-quality groundwater is frequently used for irrigation. According to Hossain (2019), the Middle East is the region most impacted by salinity, followed by Oceania and North America. Up to 50% of arable land may disappear by 2050 if the pace of salinization is allowed to continue uncontrolled (Hasanuzzaman et al., 2014). As the needs of both humans and animals for food grow, it is critical to address salinization and use breeding strategies to increase the resistance of plants to salt stress. To assess the impact of NaCl salt stress on chickpea genotypes with certain biochemical characteristics that contribute to their resilience to salt stress, R. Navyashree and V. H. Ashvathama undertook a study. When compared to other genotypes, the investigation showed that the genotype JG 11 (33.42 mg g-1 fr. wt.) had a considerably greater proline concentration at 6 dS.m-1 of salt. JG 11's high proline content allowed it to sustain low water potentials and show resistance to salt stress. After seeding, the amount of chlorophyll in chickpea plants rose for 30 to 60 days before declining. The results of the study demonstrated that in salt-susceptible plants, the total chlorophyll content of the leaves reduced during salt stress. At increasing salt levels, the loss in chlorophyll concentration was more noticeable; the greatest reduction was observed at 200 mM NaCl.

Proline and other osmolytes, in addition to their osmoprotective properties, are essential for the protection of membrane-bound proteins and enzymes. It was discovered that proline accumulation is a plant response linked to salt tolerance that aids in maintaining the proper balance between cytoplasmic and vacuolar water potential. Higher proline content accumulation in tolerable genotypes suggested that they could withstand salt stress. Ceritoğlu et al. (2020) conducted an experiment at Siirt University, using pods filled with a sand-silt soil mixture and irrigated with distilled water, 50 mM NaCl, or 100 mM NaCl. Each pot contained four seeds, and data collection included assessing germination percentage, plant height, stem diameter, number of branches, and fresh weight. Salinity stress significantly reduces chickpea seed germination, with Desi cultivars affected more than Kabuli cultivars. High salt concentrations inhibit germination by disrupting protein synthesis and gene expression, leading to over a 50% reduction in germination. In 2011, Kafi et al. carried out a study assessing chickpeas' physiological reactions to salinity stress. In a hydroponic culture using saline water at 8 and 12 dS√m–1, they cultivated 11 genotypes and compared the results with a control treatment. According to their research, proline and soluble sugars rose as salinity levels rose, but chlorophyll content and carotenoids decreased. They noticed that genotypes that contained more soluble sugars, carotenoids, and chlorophyll were better able to withstand the effects of salinity. Furthermore, a strong correlation between these characteristics and the quantities of Na+ in the leaves was discovered. Using two types (one tolerant and one sensitive) of each species, Katerji et al. (2012) exposed chick peas soy beans, grain, and barley seeds at different salinities (ranging from 0.1 to 15 dS√m–1). They concluded that there was no relationship between the plant's potential yield and its germination rate. Rather of focusing on germination rate, they recommended that emergence rate to be taken into account when assessing production potential and tolerance to salinity.

Plant height is greatly impacted by salinity stress. Low salt levels in the rhizosphere promote root growth while inhibiting the growth of aerial parts, as shown by Greenway and Munns (1980). Al-Mutawa (2003) examined how 30 genotypes of chickpeas were influenced by salinity stress for germination and growth and discovered that plant height was the trait most impacted. Reduced cellular division and cellular elongation were identified as the causes of the plant height reduction under salinity stress (Flowers et al., 2009; Shanko et al., 2017).

III. Survival Mechanism of Chickpeas Under Saline Conditions

Plants have developed a range of sensory strategies to manage salt stress, including the activation of transcription factors to control gene expression, the activation of metabolic pathways, and hormone modulation as the main signaling mechanism (War et al., 2011).

According to Deinlein et al. (2014), the plasma membrane serves as the body's first line of defense, using transmembrane sensors to detect stress. Phosphatidylcholine (PC) and phosphoric acid (PA) receptors on the root receive signals when there is a high concentration of extracellular NaCl (Jazo et al., 2011). By regulating transcription factors, PC and PA help activate the genes of the calcium-dependent protein kinase CaCDPK1, which in turn releases signaling messengers such calcium ions (Ca2+) (Syam et al. 2006; Geiger et al., 2006; Dixit et al. 2012). In order to start the transcription and translation of gene products, transcription factors attach to the gene promoter and control the RNA polymerase (Franco et al., 2014). Activation of the genes involved for osmotic adaptation has been found to depend on a number of transcription factors (Shukla et al., 2006; Peng et al., 2009; Guo et al., 2016). Some of the transcription factors that have been found in chickpea that up-regulate the CaCDPK1 genes include CAP2/AP2, CarNAC1, CaZF, and CarF (Jain et al., 2013; Jia et al., 2012). Jia et al. (2012) reported that the F-box gene CarF-box1 was identified from chickpeas treated with polyethylene glycol (PEG), simulating conditions of salt stress and drought (Jia et al., 2012). F-box gene CarF-box1 was isolated from chickpeas treated with polyethylene glycol (PEG), simulating conditions of salt stress and drought (Jia et al., 2012). Using cDNA and genomic libraries, transcription factors such as CAP2/AP2, CarNAC1, CaZF, and CarF have been found in stressed plant tissues (Jia et al., 2012, Shukla et al., 2009). (Jain et al., 2013). These transcription factors controlled the expression of genes linked to stress-induced plant growth and hormone production. Increased resistance to dehydration and salt stress has been linked to overexpression of the CAP2 gene (Shukla et al., 2006; Shukla et al., 2009).

The 202 amino acid protein known as CAP2 (for C. arietinum AP2) has an AP2/ERF domain. In vitro, the dehydration-responsive element (DRE) can bind to the Cap2 protein. According to Shukla et al. (2006), the transgenic tobacco (Nicotiana tabacum) lines that expressed CAP2 demonstrated increased constitutive gene expression that respond to abiotic stress, improved tolerance to osmotic and salinity stresses, and revealed the morphological characteristics typical of the auxin response. These findings demonstrated the interaction of two signal transduction pathways. The capacity of tobacco to withstand abiotic stress conditions and its agronomic significance are demonstrated by the expression of salinity-tolerant genes in the crop.

Under salt stress, a class of membrane-bound transporters called Na+/H+ antiporters (NHXs) help plants maintain cellular ion equilibrium (Hasegawa et al., 2013). Parveen et al. (2023) carried out research to ascertain the NHX gene expression patterns as a reaction to salinity stress.

In a factorial experiment, genotypes of chickpeas—both salt-tolerant and salt-susceptible—were examined in both normal and salt-stressed circumstances (100 mM NaCl). Following salt treatment, leaf samples were taken at various intervals for the purpose of analyzing gene expression. RNA was extracted, cDNA was created, and qRT-PCR was used to measure the expression levels of the NHX gene. The expression of CarNHX3 and CarNHX7 dramatically increased as a reaction to salt stress, especially within genotypes that are salt-tolerant, as validated by the qRT-PCR study.

IV. Using Biotechnology to Increase Salt Tolerance

4.1 Using the OsRuvb gene through genetic engineering to increase salt tolerance

Transgenic chickpea plants of the HC-1 variety bearing the OsRuvB gene were created using Agrobacterium-mediated transformation in a study by Preeti and Kharb, P. (2022) utilizing a methodology developed by Kharb et al (2012). Gene-specific primers from Eurofins Genomics, India Pvt. Ltd. were used in PCR analysis to check for the presence of the OsRuvB gene in T0 putative transgenic plants. Southern hybridization was performed using standard molecular biology methods and Thermo Fischer kits to verify the transgene's stable integration and copy number in the chickpea genome.

The transgene copy number in transgenic chickpea plants was measured using real-time PCR analysis, gene-specific primers, and the Ahmad et al. (2005) standard curve approach. Using the Phire Direct PCR kit, direct plant PCR analysis was performed to screen T1 chickpea plants for the presence of the OsRuvB gene. Together, these techniques made it possible to create and screen transgenic chickpea plants that express the OsRuvB gene, which confers resistance to salt stress (Preeti & Kharb, 2022). The OsRuvB gene was successfully inserted into transgenic chickpea plants of the HC-1 variety for the study, increasing the plants' resistance to salt stress. Reliable genetic alteration was demonstrated by the stable and single-copy insertion of the gene in these transgenic plants.

The study also demonstrated how to determine the transgene copy number in transgenic chickpea plants using real-time PCR and SYBR green. It was discovered that this technique, which enables early transgene identification during growth phases, is easy to use, effective, and economical. Using gene-specific primers, the SYBR-based test demonstrated great sensitivity and accuracy, making it a feasible substitute for Southern hybridization in transgene copy number analysis.

4.2 Genomics technologies and their application in salt stress research

Over the past decade, significant strides have been made in advancing the genomic resources of chickpea, enabling the thorough examination of the genetic basis underlying various traits crucial for tolerance and yield performance (Jacob et al. 2016; Gaur et al. 2019). Genomic advancements encompass the development of molecular markers, instrumental in assessing genetic diversity, constructing genetic maps, and identifying regions associated with desirable traits, known as quantitative trait loci (QTL) (Reddy et. al 2012). Various types of markers, such as RAPD, SSR, DArT, and SNP markers, have been developed in chickpea, facilitating the characterization of its genetic makeup (Tullu et al. 1998). The advent of next-generation sequencing (NGS) and high-throughput genotyping technologies has revolutionized data generation, enabling the capture of millions of variations at the genome level. This technological leap led to the creation of the comprehensive 'Axiom®CicerSNP Array,' containing 50,590 nonredundant SNPs, thereby enhancing genomic coverage (Roorkiwal et al. 2018).

The development of these markers has facilitated the construction of several genetic maps, including high-density ones, furthering our understanding of chickpea genomics (Kujuret al. 2015; Gaur et al 2015; Barmukh et al. 2021). Moreover, the availability of draft genome sequences, extensive resequencing efforts, and comprehensive assessments of genetic variation across cultivated and wild chickpea accessions have proven pivotal (Varshney et al. 2011, Gupta et al. 2017, Thudi et al. 2016, Varshney et al. 2013). Leveraging these genomic resources promises to enhance the precision and efficiency of breeding programs in chickpea. Through techniques like QTL and association mapping, numerous genomic regions associated with tolerance to abiotic stresses and yield components have been identified in chickpea. Major QTLs have been detected across all chickpea chromosomes, with notable genomic regions on LG3 and LG4 extensively studied for their association with tolerance to abiotic stress (Jacob et al. 2016).

Numerous studies (Mantri et al. 2007 ; Varshney et al. 2009, Garg et al. 2016, Kaashyap et al. 2018 ; Bhaskarla et al. 2020, Kumar et al. 2021) have investigated the expression patterns of genes under salinity stress conditions. These studies collectively identified 3053 differentially expressed genes in response to salt stress. Notably, in the tolerant J11 genotype, genes encoding cationic peroxidase, asparticase, NRT1/PTR, phosphatidylinositol phosphate kinase, DREB1E, and ERF were found to be significantly up-regulated (Kaashyap et al. 2018). Kumar et al. (Kumar et al. 2021), using both the tolerant J11 and ICCV 10 genotypes, identified 21,698 differentially expressed genes. Among these, 4257 genes were categorized into 64 functional groups, primarily associated with integral components of the membrane, organelles, and cellular anatomical entities. Furthermore, under salt stress conditions, significant up-regulation was observed in transcripts encoding potassium transporter family HAK/KUP proteins, MIP/aquaporin protein family, NADH dehydrogenase, pectinesterase, and PP2C family proteins (Kumar et al. 2021).

In the research study on salinity tolerance in chickpea, a genetic map was constructed using 56 polymorphic markers, including 28 simple sequence repeats (SSRs) and 28 single nucleotide polymorphisms (SNPs) . These markers were used to analyze the genetic basis of salinity tolerance in 188 recombinant inbred lines derived from the cross ICCV 2 × JG 11 (Pushpavalli et al. 2015).

The QTL analysis conducted in the study revealed two key genomic regions on CaLG05 and CaLG07 that harbored QTLs associated with salinity tolerance traits. These regions were found to influence traits related to plant vigor and reproductive success under salinity stress conditions. By identifying these QTLs, the study provided valuable insights into the genetic mechanisms underlying salinity tolerance in chickpea, paving the way for potential marker-assisted breeding strategies to enhance crop resilience to salinity stress.

V. Exogenous application to reduce salt stress

5.1 Melatonin use to reduce salt stress in chickpea

Dadasoglu et al. (2022) investigated the effects of melatonin (MT) at various concentrations (0, 50, and 100 µM) on chickpea plants facing salt stress. Under salt stress, chickpea growth was stunted, and physiological parameters like leaf water content and chlorophyll levels were negatively impacted. Additionally, salt stress induced oxidative stress, as evidenced by increased levels of hydrogen peroxide (H2O2) and malondialdehyde (MDA), while antioxidant enzyme activities rose.

Application of MT treatments ameliorated the adverse effects of salt stress on chickpea seedlings. Notably, the 100 µM MT treatment showed the most significant improvements, reducing H2O2 and MDA levels compared to untreated plants under salt stress conditions. Furthermore, MT treatments helped maintain a more favorable ion balance, with increased ratios of beneficial ions such as K+/Na+ and Ca+2/Na+.

The analysis of variance revealed significant interactions between melatonin (MT) application and salinity stress on chickpea plant growth characteristics. Salinity treatments markedly inhibited the growth of chickpea seedlings. For instance, compared to the non-salt-treated group, exposure to 150 mM NaCl resulted in reductions in plant fresh weight by 64.0%, plant dry weight by 62.2%, root fresh weight by 43.2%, root dry weight by 56.0%, plant height by 22.8%, and stem diameter by 17.3%.

Previous research has indicated that salt stress negatively impacts various physiological and biochemical aspects of plant growth (Ekinci et al. 2021 , Turan et al. 2022, Dadasoglu et al. 2021, Shams et al. 2021). Under salt stress, plants typically close or constrict their stomata to minimize water loss through transpiration, thereby limiting nutrient uptake and CO2 entry into leaf cells. This reduction in the availability of CO2 and water, coupled with irregularities in nutrient uptake, ultimately leads to a decline in photosynthetic activity, thereby impeding plant growth and potentially resulting in plant mortality (Zhanget al. 2020).

Melatonin treatments effectively mitigated salt stress in chickpea plants by improving growth and biochemical parameters, with the 100 µM concentration yielding the most promising results.

5.2 Plant growth-promoting microorganisms and biofertilizers to improve salt tolerance

It has been demonstrated by Haroon et al. (2023) that using microorganisms to reduce salt stress may effectively reduce plant stress. The aim of this study was to examine the ability of Bacillus tequilensis, a halotolerant bacterium, to mitigate salinity stress in soil by inoculation. At a concentration of 100 mM NaCl, B. tequilensis had the maximum floc production and biofilm forming capabilities, according to the research. Using Fourier-transform infrared spectroscopy, it was discovered that B. tequilensis has proteins and carbohydrates that bind to sodium ions (Na+) to provide salt tolerance (Haroon et al. 2023). Genes linked to the enhancement of plant development, including 1-aminocyclopropane-1-carboxylate deaminase and pyrroloquinoline quinone, were effectively amplified from the B. tequilensis genome using PCR analysis.

Chickpea plants under salt stress showed better physiological, biochemical, and antioxidant enzyme activity when B. tequilensis was inoculated in a saline soil environment. Relative water content was greater, photosynthetic pigment levels were higher, hydrogen peroxide (H2O2) and malondialdehyde levels were lower, and enzymatic activity for scavenging reactive oxygen species was boosted in plants treated with B. tequilensis. These results point to a sustainable application of B. tequilensis to reduce salt stress in other crops, including chickpeas. This bacteria reduces agricultural losses caused by salinity by improving plant development and mitigating the negative effects of salt (Haroon et al. 2023). For the purpose of evaluating flocculation yield potential and biofilm formation ability, Bacillus tequilensis, a strain well-known for its numerous beneficial traits, such as phosphorus solubilization, indole acetic acid production, siderophore, hydrogen cyanide (HCN) production, EPS, and ACC-deaminase activity, was chosen (Haroon et al., 2021a; Haroon et al., 2021b). This strain, which was isolated from the rhizosphere of a halophyte in the Pakistani salt mine Khewra, showed great promise. After being cultivated for four days at 30–35°C in Tryptic soy broth (TSB) medium, B. tequilensis was filtered, and the resultant flocculation was dried and weighed to ascertain the floc yield (Sadasivan and Neyra, 1985).The microtiter plate-based approach (Christensen et al., 1985) was utilized to measure the production of biofilms. The culture was cultured in TSB medium that had been modified with NaCl. The biofilm formation was then quantified by the use of crystal violet staining and spectrophotometric analysis. The shape of cells under salt stress was seen using scanning electron microscopy (SEM), and the exopolysaccharides (EPS) that B. tequilensis produces were characterized using Fourier Transform Infrared Spectroscopy (FTIR) (Haroon et al., 2021a). Furthermore, two essential plant growth-promoting (PGP) genes, acdS and pqqE, were found in the bacterial genome by PCR amplification (Naing et al., 2021; Kim et al., 2003). Lastly, a pot experiment was carried out in which B. tequilensis was injected into Kabuli chickpea seeds, which were then cultivated under regulated circumstances. After the water was treated with saline, a variety of physiological and biochemical parameters were assessed (Yasin et al., 2018; Loreto and Velikova, 2001).

According to the study's findings, Bacillus tequilensis is a halotolerant plant growth-promoting rhizobacterium (PGPR) that can improve chickpea plants' development and growth in salinity-stressed environments. By boosting the synthesis of proline, antioxidant enzymes, and total soluble sugar, Bacillus tequilensis inoculation of soil enhances plant tolerance to salt stress. According to the research, Bacillus tequilensis can be used as a useful biofertilizer in agricultural settings to lessen the effects of salt stress.

5.3 Using plant growth regulators to help chickpeas that are salt stressed

A research study was carried out at the Plant Protective Culture Unit within the semi-controlled environmental settings of the Bangladesh Agricultural Research Institute (Saif et al. 2023). The study aimed to mitigate the negative impacts of salinity stress on chickpea by applying various concentrations of salicylic acid (SA) and gibberellic acid (GA3) (Saif et al. 2023).

It involved pot cultivation of chickpea variety BARI Chola-5. Soil, exhibiting a clay loam texture with neutral pH and medium fertility, was prepared with compost and essential nutrients. Salinity stress was induced using varying concentrations of saline water, ranging from 5 to 12.5 dSm-1. Foliar sprays of salicylic acid (SA) and gibberellic acid (GA3) were administered weekly from 20 days after sowing to the flowering stage. Data on chlorophyll content, relative water content (RWC), water retention capacity (WRC), and yield parameters were collected and statistically analyzed using the R program. This randomized complete block design (RCBD) experiment aimed to evaluate the effects of salinity stress and foliar spray treatments on chickpea growth and productivity (Saif et al. 2023). The utilization of small amounts of plant growth regulators, specifically salicylic acid at 200 ppm and gibberellic acid at 10 ppm, exhibited favorable impacts on various aspects of chickpea growth. These included enhancements in pod count per plant, 100-seed weight, seed yield per plant, overall dry weight, chlorophyll content, and improvements in water-related characteristics. Particularly noteworthy was their effectiveness in alleviating the negative repercussions of mild and moderate salinity stress conditions (5 and 7.5 dSm-1), highlighting their potential for enhancing chickpea's tolerance to salinity. However, at higher concentrations, these regulators failed to significantly mitigate the adverse effects of salinity under moderate and severe stress conditions. Hence, it was found that applying plant growth regulators at low concentrations through foliar spray can counteract the decline in chickpea growth and yield caused by salinity, especially under mild to moderate stress levels (Saif et al. 2023)

VI. CONCLUSION

Salinity poses significant challenges in chickpea cultivation and therefore it is necessary to mitigate the adverse effects. Genetic and transgenic approaches, exogenous applications of substances like melatonin and plant growth regulators, as well as the use of plant growth-promoting microorganisms and biofertilizers, show promise in enhancing salt tolerance. Furthermore, exogenous applications of substances such as melatonin and plant growth regulators have demonstrated efficacy in ameliorating salt stress effects, improving physiological parameters and antioxidant enzyme activities in chickpeas. Advancements in genomics technologies have facilitated the identification of quantitative trait loci (QTLs) associated with salt tolerance, providing valuable resources for marker-assisted breeding efforts to develop resilient chickpea cultivars. Moreover, the utilization of plant growth-promoting microorganisms and biofertilizers has emerged as a sustainable strategy to mitigate salt stress, enhancing plant growth and development in saline environments. A multi-faceted approach integrating these strategies is essential for sustainable chickpea production amidst escalating environmental pressures.

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