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BIOTECH FOR THE MAJOR COMMERCIAL CROPS

Cotton, Maize, Potato, Sugarcane

(with a special focus on PGPR)

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About this Book

This book takes an in-depth look at the economic importance, challenges, and biotechnological innovations impacting major agricultural crops like cotton, maize, potatoes, and sugarcane based on the lifelong work of the editor and his various research groups. Spanning five comprehensive chapters, the text explores how genetic engineering, pest management strategies, and sustainable agricultural practices can enhance crop productivity, quality, and resistance.

The opening chapter discusses the economic significance of cotton production and the critical role of transgenic approaches in combating insect pests and weeds. It examines the benefits of Bt and glyphosate-resistant genes, as well as advancements in RNAi and viral resistance for tackling cotton leaf curl disease. The maize chapter subsequently covers pest and weed management, development of insect-resistant transgenics, and the potential of chitinase for sustainable pest control.

A full section is devoted to the role of plant growth-promoting rhizobacteria (PGPR) in improving soil nutrient availability and crop yields across various agricultural systems. The text characterizes phosphate-solubilizing bacteria and explores their applications for enhancing sugarcane growth, alleviating salinity stress, and supporting sustainable practices.

The potato chapter explore RNA silencing strategies and genetic engineering for achieving viral immunity, while also addressing mechanisms and innovations in disease resistance more broadly. Finally, the sugarcane segment explores genetic engineering for disease and virus resistance, herbicide tolerance, and optimizing

sucrose production and sugar isomerization through microbial applications.

Featuring the latest research and case studies, this comprehensive volume equips readers with a thorough understanding of how biotechnology and sustainable agriculture can address the mounting challenges facing major crop production worldwide.

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Chapter 1: Cotton

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Economic Importance of Cotton and Challenges from Insect Pests and Weeds

Cotton is one of the most important cash crops; it supports, directly or indirectly, millions of livelihoods in many countries around the world. Cotton is a backbone of the economy in Pakistan, availing much employment and raw materials to industries. However, the crop has to bear different stresses which badly hamper its productivity, and one of the most impending stresses is the threat from insect pests, leading to huge losses. An estimated $216 million of the $645 million lost to yield losses caused by insects each year is attributable to *Lepidopteran* insects. The pink bollworm, scientifically known as *Pectinophora gossypiella*, is a particularly troublesome *Lepidopteran* pest in the US. It damages a tiny fraction of cotton fields but is responsible for an estimated $71 million in losses. Insect pest management methods that rely on human intervention are time-consuming and costly for modern farmers. Farmers typically spray their fields with chemical pesticides six or seven times per season as a result of insect infestations. Nevertheless, there are a few drawbacks to chemical pesticides that have come to light. These include their high costs, the fact that they stay in the environment, and the fact that they need to be applied more frequently since they become less effective with time.

Because they compete with the crop for growing resources, weeds are another key factor that typically affects cotton yield. Hoeing weeds by hand is the most common approach, but it is extremely labor- and time-consuming, therefore modern farmers have done away with it. The overall accumulation of weed seeds in the soil is significantly increased when non-chemical weed control approaches are used instead of chemical ones. When compared to non-chemical

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approaches, biotechnology stands out as a viable option for addressing this issue. One of the most used herbicides, glyphosate (N-phosphonomethylglycine) kills a wide variety of grasses and annual broadleaf weeds that compete with agricultural plants all over the world.

The vital cash crop that supports farmers' and labourers' livelihoods is cotton. It supplies over 80% of the raw materials needed by various businesses, and as a result, cotton accounts for about 30% of Pakistan's foreign exchange revenues. Cotton output is influenced by and decreased by several things. Among these is the rivalry for resources between Weeds and cotton plants are competitors in addition to insect pest attacks. Insects cause roughly 20% of agricultural losses, while weeds are thought to contribute about 25% of crop loss overall.

Gossypium hirsutum L., the scientific name for cotton, is an extremely valued crop with significant commercial value. Cotton plant seeds yield a multitude of important items, such as oil, feed, and fiber. Pakistan's textile sector is worth roughly 2% of the country's GDP, exporting second-most cotton yarn and producing seventh-most fabrics worldwide. For this reason, the cotton industry is critical to the economy and the millions of workers whose jobs depend on it during the growing and processing stages. Cotton crops are seriously threatened by *Lepidopteran* pests such the Pink Bollworm (*Pectinophora gossypiella*), Army Bollworm (*Spodoptera litura*), American Bollworm (*Heliothis armigera*), and Spotted Bollworm (*Earias insulana/vitella*). Pest management has traditionally involved the use of strong chemical pesticides. However, because these pesticides damage plants and represent major environmental risks, this approved practice has proven to be unsustainable.

Assessment of PME Gene Expression in Transgenic Cotton Lines

To determine the relative mRNA expression of the PME gene in the transgenic cotton plants, we employed real-time PCR. In comparison to line PME11, the plant with the lowest transgene mRNA expression levels in the study, the relative expression of the AnPME gene was three times higher in the transgenic line PME5. The findings align with existing literature demonstrating that different lines within a single

transgenic cotton population display varying degrees of transgene expression. As the transgenic expression levels in all lines under investigation varied noticeably, line PME5's transcript levels of the AtPME gene were 3.5 times higher than line PME11's.

Despite being a commodity of significant economic importance, cotton is controversial since it harbors the bulk of weeds, viruses, and insect pests. Insect insecticides have historically been used to manage insect pests, but the results have typically led to financial losses that have been felt in various areas. Although traditional breeding techniques have improved cotton significantly, they have intrinsic limits on the introduction of new alleles, particularly for responses to more recent problems like pest stresses. From this point, too great a reliance upon insecticides in developing countries raises fears concerning environmental sustainability and economic viability. In light of these conditions, biotechnology is a developing substitute for traditional breeding techniques in the development of genetically modified cotton types that are both environmentally and commercially advantageous. Because cotton biotechnology has the ability to completely change the culture around cotton agriculture, it has enormous commercial consequences. The commercial introduction of cotton, one of the first genetically modified crops, encourages the acceptance and integration of transgenic technology into agricultural production systems. This indicates that growers have created alternate strategies to get over the main obstacles that could wipe out cotton crops in an effort to increase profits. One of the biggest problems farmers have is weeds competing with crops for nutrients and water, which lowers yield quality and quantity. The traditional method of managing weeds involves mechanical uprooting or manual labor, in which farmers use tearing-type tools to uproot undesirable plants.

The weed management strategies used in crop production has fundamentally changed as a result of the discovery and adoption of herbicide-resistant crops. In order to provide crop plants with resistance against glyphosate, genetic transformation has introduced bacterial genes producing 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) into the plants. This change makes it possible to apply glyphosate, the most widely used herbicide, on crops without harming them while controlling weed populations. Furthermore, transgenic cotton may express crystalline proteins derived from genes of the Gram-positive bacterium *Bacillus thuringiensis*, which are harmful to

the larvae of various insect orders, including the Lepidoptera, Coleoptera, and Diptera. Thankfully, these genes have previously been used to create insect-resistant crops, and transgenic cotton that expresses *Bacillus thuringiensis* insecticidal proteins has become widely accepted worldwide.

Economic and Environmental Benefits of Bt and Glyphosate-Resistant Gene

One or more Cry genes effectively control *Lepidopteran* pests in these genetically modified cotton cultivars, also known as Bt cotton. Growers have seen substantial economic and environmental benefits from using Bt cotton, as the use of chemical pesticides has been greatly reduced. On top of that, it has managed to keep vital arthropod populations intact. Recent studies have mostly focused on Agrobacterium-mediated gene transformation into local varieties such as VH289. A glyphosate-resistant gene (cp4EPSPS) is fused with two Bt genes (Cry1Ac and Cry2A) in this gene transformation. The herbicide-resistant gene and the Bt genes were driven during cloning by a constitutively expressed CaMV 35S promoter. The purpose of this genetic modification was to confer insecticidal and herbicide resistance on the cotton variety VH289. A carefully selected promoter, CaMV 35S, produces transgenes strongly and consistently in green plant tissues but only weakly in non-target tissues like roots. This is important from a biosafety standpoint since it reduces the impact on soil microbes and the environment.

Cotton output is the end goal as a crop plant, but weeds, viral infections, and insect pests are the main enemies of cotton productivity. Recent studies have shown that in order to solve these issues, genetically modified cotton is required, specifically cotton containing the cp4EPSPS gene, codon-optimized Cry1Ac and Cry2A genes. Prior studies have shown that the Cry1Ac gene can be utilized to shield cotton against insect pests, and comparable approaches have also been employed to control weeds using genetic engineering. The capacity to incorporate numerous advantageous features into a single cotton variety is only one of many benefits that have prompted discussions of genetic modifications in this setting, which offer several benefits over conventional breeding techniques. An insect- and weed-resistant cotton variety is the end aim of the gene pyramiding approach. The process comprises simultaneously inserting the native

cotton variety VH 289 with a glyphosate tolerance gene, cry1Ac, and cry2A, two Bt genes. Roundup, whose active ingredient is glyphosate, is a broadleaf crop and weed killer that does not selectively target any one type of plant. Therefore, it is essential for weed control in agricultural areas.

Biotechnological Approaches to Enhancing Pest Resistance in Cotton

In view of these problems, insecticidal toxin engineering from the soil-dwelling bacterium *Bacillus thuringiensis* has become a competitive alternative for pest management. Although these Bt toxins were effective in their early application, the development of resistance amongst the pest population due to Bt δ-endotoxins has developed as a challenge. An alternative approach to this growing resistance includes an increase in synthesizing capacity by the plant for certain defense-related enzymes, helping to complement the efficacy of Bt toxins and reducing the risk of resistance development. This could be accomplished through the overexpression of endogenous genes responsible for defense mechanisms in cotton.

Because animals lack the EPSP synthase-catalyzed pathway, the herbicide's selectivity and safety profile are highlighted. Weed management in agriculture has undergone a radical transformation since its incorporation into transgenic crops. The reliance on GR crops, which saw a dramatic growth in the land dedicated to these transgenic kinds, began with its initial commercial release in 1997. The glyphosate-resistant gene has shown encouraging results when introduced into native cotton varieties in many research settings, including China. For example, by utilizing the hypocotyl of CRI 35 as an explant, a group of researchers successfully propagated 65 plants. In addition to carrying the insect-resistant gene Bts1m, the other inserted gene, aroAM12, makes the cotton genotype Shiyuan-321 resistant to glyphosate. Molecular investigation showed that 38 of the 52 regenerated plants that were produced by this Agrobacterium-mediated transformation harbored both transgenes. Concurrently, the biolistic gun's capacity to introduce the Cp4 EPSPS gene into soybean showed how flexible gene transfer techniques are, opening the door to the possibility of producing herbicide-tolerant crops that don't sacrifice yield. Actually, GR crop cultivation is essential since weed competition

drastically lowers cotton yield, particularly in the early growth stages. It has been demonstrated that yield losses in cotton can reach 30% when weed pressure is the sole factor. This proves without a reasonable doubt that an effective weed management strategy is necessary for maximum crop production. Traditionally, methods of management included crop rotation, polyculture, and hand weeding.

On the other hand, the 1930s saw the strategic change towards more focused and effective weed control techniques with the development of synthetic herbicides. Crop management has advanced strategically with the creation of transgenic cotton plants that express the codon-optimized CEMB GTGene. To enhance transgenic expression in *Gossypium hirsutum*, which imparts extra resistance traits to cotton varieties, codon optimization was used. Using restriction digestion with NcoI and BglII enzymes, the codon-optimized cp4EPSPS gene was cloned into the pCAMBIA 1301 vector after optimization. Subsequently, Agrobacterium was introduced into mature cotton embryos in order to transform the plants and successfully incorporate the transgene into the cotton genome.

Genetic Engineering for Enhanced Pest and Weed Resistance in Cotton

It inhibits the shikimic acid metabolic pathway, which is necessary for the synthesis of EPSPS, which consists of the three aromatic amino acids tryptophan, phenylalanine, and tyrosine. Plant genetic engineering improves weed management and confers resistance to diseases, insects, and weeds by modifying plant genes—all without using conventional breeding methods. Some have also mentioned that genes that code for antifungal proteins, including as endochitinase, glucose oxidase, and β-1,3-glucanases, have been successfully integrated, along with components of signaling networks related to the plant defensive response. Several transgenic plants with resistance to a wide range of plant diseases have been created using these various techniques.

The screening and selection for the elites of cotton and other types following genetic transformation is an essential step in developing highly successful genetically altered plants. Potentially reducing pest management costs while boosting overall production. Cotton varieties

with sustained transgenic expression and high transformation efficiency could be worth exploring. Various methods are now under development to enable the modification of certain genes through genetic engineering. The study aimed to modify three target genes—cp4EPSPS, which is a herbicide resistance gene, and Cry1Ac and Cry2A, which are CEMB double Bt genes—in order to control weeds and insects simultaneously. The goal of is to find out which cotton cultivars can withstand biotic challenges the best by undergoing genetic transformation, transgene acclimatization, and sustained expression.

Within this framework, pectin methylesterase is of interest to us because it catalyzes the demethylation of pectins in plant cell walls. This consists of methanol produced during this action and is important in the defense of the plant against herbivorous species. Previous studies indicated that attacks by herbivores induce activity of PME in most plant species, followed by emission of methanol. Methanol is toxic to insects; thus, it would appear considerably likely that its accumulation increases insect toxicity within the host plant tissues. Insect-feeding, damaged, and wounded plant tissues have been considered as a key source of production of methanol mediated by an enzyme known as PME.

Enhanced Insect Resistance through PME Gene Overexpression

The current research looked at transgenic cotton plants that have PME genes overexpressed from two separate sources: *Arabidopsis thaliana* (AtPME) and *Aspergillus niger* (AnPME). We wanted to see how changing the expression of PME genes affected insect resistance; therefore we used AtPME and AnPME for that purpose. The need for better cotton varieties is being pushed by shifting market dynamics and the crop's ongoing industrial importance, which calls for creative approaches in plant biotechnology. Enabling the transfer of genes from multiple unrelated sources into cotton is one of the main objectives of genetic engineering in these sectors, as it will boost cotton's resilience to a variety of insect pests. By combining two Bt genes into a single construct and inserting PME genes from *A. thaliana* and *A. niger* into *G. hirsutum*, we took a unique strategy to solving this issue. We were able to produce transgenic cotton lines that are resistant to a wide range of insects as a result. This technique was developed to solve issues

related to insect populations becoming resistant, which surfaced in earlier studies employing non-transgenic tobacco. The PME gene was cloned into the vector pCAMBIA 1301, and then it was introduced into *G. hirsutum* by means of Agrobacterium-mediated transformation. A calculated transformation efficiency of about 1.32% agrees with figures reported in the literature. The first transgenic cotton plants were selected using hygromycin selection. Their transgenic status was further validated and precise identification of the transgenic events was made possible by the use of gene-specific primers in PCR analysis. Subsequently, leaf bioassays were carried out to ascertain the transgenic plants' efficacy against insect pests and further investigations to ascertain the transgene's mRNA expression in the PCR-positive plants.

Methanol Production in Transgenic Cotton

There has never been any evidence that plants can be damaged by methanol, a metabolite that plants make. Rather, it builds up in leaves and then is discharged into the air through stomatal holes, where it serves as a natural defense against insects. Using a state-of-the-art mass spectrometry method, studies estimated the methanol level in cotton plants that were either transgenic or non-transgenic. According to the results, compared to the relevant non-transgenic control line, which had a methanol concentration of 0.1%, the transgenic lines PME4 and PME5 had an elevated methanol concentration of around 0.8 and0.9%, respectively. The methanol concentrations in PME3 and PME11 were 0.5% and 0.6%, respectively, showing that these lines were reduced compared to PME4 and PME5, but elevated compared to the non-transgenic control. Since transgenic lines PME4 and PME5, which express high levels of both PME genes, killed off all of the pests in the insect bioassay, which corroborated the findings of the methanol content increase.

The insects were tested in bioassays with things like flowers, leaves, and cotton bolls. A transgenic cotton plant carrying the AtPME and AnPME genes in addition to the two Bt genes was inoculated with second-stage *H. armigera* larvae. By the fifth day of the experiment, 82% of the control plants had died out, whereas transgenic cotton lines PME4 and PME5 had nearly full mortality at 100%. In a similar vein, insects were still active seven days into the test, indicating that the

non-transgenic control plants had minimal mortality rates for larvae feeding on them.

An alternate bioassay involved introducing newly-grown bolls of the transgenic cotton variety CEMB-33 to Pink Bollworm (*P. gossypiella*) larvae. Plants resistant to Pink Bollworm had emerged in three days, despite the pest killing sixty-three percent of plants in lines harboring the two Bt genes along with the AtPME and AnPME genes. The transgenic cotton plants with only two Bt genes exhibited a 50% death rate as opposed to the non-transgenic control plants, which had a 0% mortality rate. In this research, we have demonstrated that transgenic cotton plants can become more insect-resistant by overexpressing the PME genes from *A. thaliana* and *A. niger*. The findings have illuminated the complex relationships between insect populations and plant resistance, offering hope for the creation of novel transgenic crops that could fight unwanted pest infestations without harming the ecosystem like traditional pest control techniques do. Such genetic approaches could lead to improved cotton yield and quality, as well as increased resistance to evolving insect threats. The investigation's findings demonstrate that overproducing methanol is a feasible substitute for employing insect vectors that have grown resistant to Bt proteins. Pakistan's agriculture industry contributes more than 21.4% of the country's GDP, making it a significant economic engine. Within this industry, cotton contributes 1.2% of GDP and more than 60% of the nation's foreign exchange earnings. Nonetheless, Pakistan's cotton production is seriously threatened by Cotton Leaf Curl Disease. Prior to 1986, this condition was rather uncommon. But because of the impacts in the years that followed, it turned into an epidemic in 1991 and 1992, which resulted in large yield losses at that time. CLCuD has been mainly under control since resistant cotton types were released in the late 1990s. However, the Cotton Leaf Curl Burewala virus, now known as the Cotton Leaf Curl Kokhran virus-Burewala strain (CLCuKoV-Bur), was responsible for a second epidemic that struck the Burewala district of Punjab in 2001 and 2002. This virus strain then spread to most of Pakistan's cloth-growing regions.

Advancements and Challenges in Bt Gene Integration

Pesticide usage in Bt cotton fields in India has been shown to drop by as much as 70%, with significant savings in insecticides and an

astounding 80–87% rise in cotton yields. Despite the success of cloning and transgenic plant genes including Bt, the insecticidal effectiveness of the resulting plants is typically diminished. One possible explanation for the decrease in performance could be that target insects have developed resistance to the produced Bt proteins. This is something that is being observed more and more in applications involving single Cry genes.

For these reasons, scientists have been trying to create transgenic plants that can express insecticidal genes from bacteria by penetrating chloroplasts with cry genes, modifying bacterial genes to use plant-preferred codons, and expressing these genes in chloroplasts through transit peptides. A new development in the field of transformation is the expression of transgenes in specific locations by means of chloroplast transformation. There are significant benefits to using this method for tissue-specific gene expression in plant green tissues; nevertheless, so far, it has only been applied to *Solanaceae* plants. The majority of studies examining chloroplast transformation have focused on tobacco since it is one of the few plant species that thrives when transformed using biolistic or Agrobacterium-mediated methods in tissue culture. A major limitation of chloroplast transformation technology in cotton is the difficulty in regenerating the crop on tissue culture media due to the cotton plant's notoriously stubborn nature. The DNA found in chloroplasts codes for just 10% of the proteins needed; the other 90% are brought in from the cytoplasm by means of specific trans-peptide signals. If precursor proteins could be transported into the chloroplast by connecting transit peptides to the N-terminal of the Bt gene, the cotton nuclear transformation may be improved. Science has proven that *Lepidopteran* insects can evolve a resistance to BT crops that carry just one Cry gene. This realization underlines the pressing need for new strategies that deploy multiple lines of defenses with the object of retarding resistance development in insect populations.

The primary goals of this research are to understand how to use the Cry1Ac and Cry2A genes to increase plant resistance and find a way to use nuclear transformation to target expression to chloroplasts, thus overcoming the difficulties of tissue culture in cotton. Stable gene integration allows plant chloroplasts to express target proteins, which in turn can result in high yields. The results of chloroplast transformation revealed that a significant amount of foreign proteins

accumulate, reaching as high as 46% of the total protein in the leaves. Because each chloroplast cell contains an extremely high copy number of the chloroplast genome, chloroplast transgenic plants are able to express a plethora of functional genes. Another benefit of chloroplast transformation is high expression levels, which are 169 times higher than those from nuclear transformations. This is in addition to the fact that it reduces the risk of transgene silencing. Another advantage of chloroplast-targeted engineering is that it allows transgene stacking, which means multiple transgenes can be expressed simultaneously. The ability to generate multivalent expressions in a single transformation step is a boon to pest resistance strategies in general. A fusion protein with the chloroplast transit peptide at its N-terminus and a Bt component at its C-terminus promotes resistance to *Lepidopteran* insects through enhanced expression levels. The idea behind this strategy is to use cutting-edge genetic engineering techniques to generate transgenic crops that can withstand biotic challenges, including pest insects. At the vanguard of current research towards the creation of sustainable agricultural solutions is this effort, which integrates diverse resistance strategies with the benefits of chloroplast transformation. Through rigorous research and development, they will significantly increase cotton production by making it more resistant to viral infections and insect attacks, allowing agriculture in cotton-based countries to remain sustainable. The production of the *Bacillus thuringiensis* gene specifically targeting the chloroplasts is one possible method for engineering plants to acquire important agronomic features. Plants are able to produce crystal proteins that are toxic to numerous insect species by utilizing the abundant Bt gene expression in chloroplasts. Presently, we are developing a strategy to insert a chloroplast transit peptide-linked pair of insecticidal genes, Cry1Ac and Cry2Ab, into the cotton MNH-786 plant using the plant expression vector pBI-121. Cry1Ac and Cry2Ab were chosen as insecticides because to their distinct characteristics, such as their high expression levels and absence of competition for receptor binding sites. Results show that cloning genes with transit peptides is a powerful tool for improving cotton plant expression.

Using the Hind III restriction site, the Cry1Ac and Cry2A genes were successfully cloned into the pBI-121 vector after being provided with a chloroplast transit peptide. This allowed for successful gene integration. The use of gene-specific primers in conjunction with orientation primers allowed for the verification of successful cloning.

To test the cloned genes' activity, researchers utilized an agro-infiltration experiment, which revealed that the genes had been expressed when they caused a bluish-green discoloration in the infiltrated areas. This finding is consistent with previous research on similar works and lends credence to the validity of the methodology. In addition to addressing any health and biosafety concerns associated with transgenic plant products, targeting the localization of transgene proteins in green plant tissues is achieved through the transformation of Cry genes with transit peptides.
The expression of transgenic proteins was studied using both quantitative and qualitative methods.

Together with the control sample, the transgenic plants showed a band in the dipstick assay, proving that the target protein was present and lending credence to the idea that the transgene had been introduced and expressed properly. After conducting the ELISA assay on Cry proteins, it was found that the Muz-01 line contained the highest amounts of Cry1Ac and Cry2A proteins among the transgenic plants, with values of 0.673 ng and 0.454 ng, respectively. Cry protein expression was not detectable in the control set of plants, further demonstrating the success of the transformation.

Integrating Bt Proteins and RNAi

Fluorescence microscopy with FITC staining techniques accredited the successful integration of Cry proteins into the chloroplasts. Earlier research has also reported successful expression of proteins in chloroplasts and demonstrates that this methodology has been effective in this research. Insect bioassays performed afterwards indicated higher mortality rates in the American bollworm, a major cotton pest. Field trials done to assess the effectiveness of the transgene agreed with this, since in the insects feeding on the leaves of the transgenic cotton, a whopping 100% mortality was recorded. These observations, therefore, agree with earlier published studies and undermine effectiveness in a buffet style for the control of pest populations using the transgenic cotton. The results indicate that cloning of multiple genes such as fusion genes, in addition to adding transit peptides for subcellular localization into the chloroplast, greatly enhances the effectiveness of Bt proteins in insecticidal applications. The technology also solves some biosafety issues associated with the

deployment of transgenic crops. The molecular analysis from the entire study leads to the conclusion that among all, those transgenic plants whose dual Bt genes were fused with a chloroplast transit peptide showed better resistance against *Lepidopteran* insects than the plants expressing genes without any fusion of transit peptide.

The wider ramifications of viral infections on cotton output need to be thought about in light of these developments in transgenic insect resistance. Cotton and other members of the Malvaceae family are naturally infected with a variety of begomoviral species and strains, one of which is CLCuD, a severe disease. Since the early 1990s, Pakistan has suffered agricultural losses of 5–30%, or more, due to the leaf curl disease, which has resulted in losses estimated at over US$ 5 billion. Damage to almost 1.48 million hectares of cotton crop area and losses of about 1.12 million bales were incurred during the severe onslaught that occurred in 2008 and 2009. Despite being a top six exporter and number four producer of cotton globally, Pakistan falls to near the bottom of the pack when yield per acre is considered. As a result, the country imports 1.5 to 2 million bales of cotton annually to satisfy its textile industry's demand. Reducing feeding damage caused by *Lepidopteran* pests has been achieved through the application of transgenic techniques based on the expression of Bt-toxin. A number of issues arose during the commercialisation of transgenic technology for the control of viral diseases. There is a lot of evidence that gene silencing can control viral infections by preventing pathogen-derived resistance mechanisms, however these technologies are not used holistically. Above all else, dsRNA-mediated RNAi has emerged as a powerful method for inhibiting viral gene expression in plants, opening up new possibilities for disease resistance, particularly against Geminiviruses.

Applying short interfering RNA technology, which targets coding and non-coding regions critical for viral replication and movement inside the host, has resulted in the stable suppression of viral gene expression. Systemic RNAi knockdown impacts a plant's capacity to respond to a silencing signal. The resistance profile can be elevated in some cases when dsRNA can be exported to other cells. Because of this art, we may use fewer chemical pesticides to manage insect vectors, particularly the whitefly, which is a good vector for Begomovirus*es*. A promising opportunity to develop transgenic crops resistant to viruses

by expressing double-stranded RNA (dsRNA) is presented by RNA interference.

Begomovirus*es* attack plants worldwide, whether they are wild or domesticated. These little viruses belong to the Geminiviridae family and have DNA that is only one strand long. There are two types of viral genomes: bipartite, which contains both DNA-A and DNA-B, and monopartite, which contains only DNA-A. Most unusual monopartite Begomovirus*es* can only infect plants systemically if they attach a betasatellite to themselves. One reason for this is that the betasatellite helps the virus infect more cells by suppressing the host plant's defences. So, the AC1 gene is essential for Begomovirus regulation of viral DNA transcription and replication since it encodes a replication-related protein. Following the injection of a nick, replication at the viral DNA origin occurs; this process is critically dependent on this multifunctional protein that resides in the plant nucleus. The connections between the Rep-associated protein and sequences in the satellite-conserved region facilitate betasatellite replication. Sharing a nonanucleotide sequence, the SCR and its related helper virus seem to rely on this region to initiate betasatellite replication. It appears that the SCR's location on the DNA molecule is comparable to the common region typically seen in bipartite Begomovirus*es*, further complicating viral interactions within infected plants. Using RNA interference and Bt gene expression targeted to chloroplasts could significantly increase cotton's resilience to viruses and pests. In addition to helping address major biosafety issues with transgenic crops; this will enhance agronomically important traits of cotton varieties. As researchers delve further into the complex web of relationships between plants, viruses, and insects, a more sustainable agricultural structure that prioritizes both productivity and environmental friendliness becomes increasingly likely. This makes us hopeful for the development of tough cotton cultivars that will be able to resist these challenges. Some believe that gonomoviruses use satellites in some way to regulate their virulence. Furthermore, these satellites impact the severity of symptoms and illness inflicted upon plants by Begomovirus*es*. When the helper virus infects plants with DNA-A without the DNA β satellite, there is a marked decrease in virus accumulation compared to when wild type viruses are present. So, when the viral load is reduced, most persons experience mild symptoms or none at all. On DNA satellites, you'll only find one coding gene called beta C1, or bC1. It is able to prevent genes from being silenced after transcription by producing a protein. The

pathogenicity of Begomovirus*es* is believed to be largely dependent on this form of suppression. This was anticipated since blocking the expression of the bC1 gene inhibits Begomovirus from infecting most host plants systemically.

RNA Interference and V2 Gene Targeting in Developing CLCuD

In order to conduct this work, scientists modified two premium cotton types, MNH-786 and VH-289, with an RNAi construct that targets the V2 gene of the CLCuKoV-Bur virus. Silencing the V2 gene reduced the virus's ability to spread among plants. The same has been attempted in Indian varieties of cotton using RNAi constructs targeting the V2 gene and intergenic region. Due to the fact that cotton is "white gold" for Pakistan, economic importance imparts urgency for enhancement of crop production, its betterment regarding fibre quality and its pest management through its genetic alteration. The resistant varieties of crops combined with the use of insecticides have so far been the main method to control CLCuD. It is known that through historical data, resistant cotton varieties were developed in the 1990s. However, these varieties lost their effectiveness as a result of fast evolution of the virus.

Given these challenges, RNAi presents a viable alternative strategy for controlling CLCuD. Transgenic cotton plants may be able to resist CLCuKoV-Bur CLCuD; amplicon V2 RNAi targeting the highly conserved V2 gene of the Begomovirus may be the key. When exposed to viruliferous white flies in controlled conditions, the majority of transgenic cotton plants displayed fewer disease indicators and lower disease index ratings than their wild-type counterparts. In quantitative PCR investigations, the presence of the virus was detected in all transgenic plants; however, the viral titers differed significantly from those of the non-transgenic cotton plants. More properly, the resistance that this technology offers can be described as a high tolerance of infection; in other words, even in cases when the plants are infected, they are able to effectively mitigate the negative effects of the virus on their well-being and yield. Our results support the use of RNA interference (RNAi) to produce transgenic resistance to several Geminiviruses, in line with previous studies. MicroRNA methods can efficiently defend plants against monopartite Begomovirus*es*, which is

another indication of the adaptability and promise of RNA interference in crop security. The goal of this work was to deactivate the V2 protein, which, when expressed in plants via vectors like Potato Virus X, is in charge of defining symptoms and causing programmed cell death. In order to accomplish this, the expression vector was used to introduce RNA interference of the amplicon V2 gene into the elite cotton varieties MNH-786 and VH-289. The fact that V2 protein suppresses gene silence further highlights how important it is to concentrate on this protein in order to develop superior cotton varieties that are disease-resistant. Recent developments in our knowledge of the molecular mechanism behind virus-plant interaction and the successful application of RNA interference (RNAi) technology in transgenic crops have accelerated the development of effective strategies for regulating CLCuD. Hopefully, in the near future, new findings and developments in plant biotechnology may result in the development of cotton varieties resistant to these viral infections, assisting Pakistan's cotton sector in staying viable and stable economically. This paper highlights the convergence of biotechnology and agricultural science to tackle some of the most critical pest management issues in contemporary crop production and the upcoming GMO era. It's common knowledge that the V2 protein, which the Cotton Leaf Curl Multan Virus encodes, has potent suppressor capabilities for gene silencing. It is possible to genetically modify tobacco plants to produce an antisense construct that specifically targets the Tomato Leaf Curl New Delhi Virus's AV2 gene. This has already been shown. These transgenic plants had infectious clones of ToLCNDV inserted into them; however they did not exhibit any disease signs. However, PCR analysis proved the presence of their viral DNA. In a different experiment, the Indian cotton variety F-846's V2 genes of CLCuKoV were modified via RNA interference (RNAi) and Agrobacterium-mediated transformation. The resultant transgenic cotton plants showed resistance to the complex CLCuD virus and a Mendelian inheritance pattern. This work offers a thorough examination of the resistance offered by the amplicon V2 RNAi construct in addition to describing the process of creating transgenic cotton plants. We could not find any indication that transgenic cotton plants using this amplicon V2 RNAi technique might be given high tolerance levels. Furthermore, the resistance to viral infection can be strengthened by combining amplicon V2 RNAi with other tolerance and resistance mechanisms.

Advances in Cotton Leaf Curl Disease Management

The hallmark symptoms of CLCuD include enation formation, which are cup-shaped growths on the underside of leaves; thickness, darkening, and swelling of veins; and upward or downward curling of leaves. The unusual complex of many monopartite Begomovirus*es* from the Geminiviridae family that causes CLCuD requires a satellite molecule known as the CLCuMB. The bC1 protein that the Begomovirus produces makes it a pathogenicity determinant in and of itself, capable of overcoming host defensive mechanisms, even though it is necessary for CLCuMB's replication and encapsidation. Remarkably, it has been discovered that CLCuKoV-Bur is a recombinant virus that unites the Cotton Leaf Curl Kokhran and the Cotton Leaf Curl Multan strains. Furthermore, the beta satellite associated with CLCuKoV is, in reality, a combination of two other beta satellites: Tomato Leaf Curl and CLCuMB. There is also evidence connecting the virus to the alpha satellite, a self-replicating molecule. Although the precise function of the alpha satellite is yet unknown, research has demonstrated that the rep protein expressed by an alpha satellite reduces gene silencing. *Bemisia tabaci*, the whitefly vector, carries these viral components to the cotton plant.

No cotton genotypes have been found to have a natural resistance to CLCuD as of yet. Here, agronomically-grounded CLCuD-tolerant varieties may provide a bid from biotechnological methods, the primary RNAi techniques. Down-regulating target genes in a sequence-specific way are the end result of RNA interference, a gene silencing process based on homology. Begomovirus*es* are successfully targeted at two levels by this process: the transcriptional level, through viral DNA methylation, and the post-transcriptional level, through viral transcript destruction. In RNA interference (RNAi), dsRNA is used as a starting point. An enzyme called Dicer-like cleaves it into short interfering RNAs (siRNAs), and each siRNA directs the process of sequence-specific silence. The RNA-induced silencing complex (RISC) is involved in PTGS, where the siRNAs aid in the destruction of homologous messenger RNAs.

Plants can develop virus resistance by expressing hairpin constructions with sequences that are identical to the virus of interest. The ability of the RNA interference (RNAi) signal to propagate from one cell to another, as well as to distant tissues, is a major benefit of this

technology. Insect-resistant genetically modified plants have recently gained attention thanks to RNAi technology. The introduction of RNAi mechanisms against certain insect pests has led to the production of transgenic crops such as cotton, rice, and maize, with other GM crops currently under research. Using RNA interference (RNAi), a few scientists were able to create a vaccine that was resistant to Geminiviruses. Among their most notable accomplishments was the development of RNAi-mediated bean resistance to the Bean Golden Mosaic Virus in Brazil.

Constructs in Reducing Viral Load in Transgenic Cotton Against CLCuD

The development of a dsRNA construct was an integral part of this investigation. As part of it, there was a cloned AC1 gene segment from the Cotton leaf curl Kokhran virus-Burewala (CLCuKoV-Bu) and a bC1 coding segment that overlaps with the non-coding SCR of the Cotton leaf curl Multan betastatellite (CLCuMB). To ensure the resulting fragments were intact, DNA sequencing was performed after sub-cloning. After then, it was modified into the *Gossypium hirsutu* variation so it could be cultivated in agricultural environments in Pakistan.

This work set out to genetically alter the VH-289 cotton variety using a dsRNA hairpin construct named V_. Two genes—CLCuKoV-Bu AV1, which is critical for viral replication, and bC1, along with the SCR region of CLCuMB—were intended to be silenced or knocked down by this construct. To achieve this transformation, the Agrobacterium-mediated embryo shoot apex cut method was employed. This approach had been fine-tuned for usage in other studies with comparable objectives. After successfully introducing the V_ construct into transgenic cotton plants, beta satellite accumulation was significantly reduced. The T0 and T1 generation cotton lines were found to contain the transgene construct as confirmed by PCR amplification of a 300 base pair fragment of the CLCuKoV AC1 gene. This gene is crucial for starting the replication of the Begomovirus and its associated beta satellite.

After confirming by FISH and karyotyping that the transgene was integrated as a single copy on chromosome 6, the asymptomatic V_6

transgenic plant line had the lowest viral burden in T1 generation plants. This result distinguishes out from the usual, which is random integration with varying copy numbers, when Agrobacterium-mediated transformation is used.

To determine if transgenic mice could withstand CLCuKoV-Bu and its companion virus, CLCuMB, we conducted field tests on two generations of mice. The Begomovirus and its associated satellite were successfully suppressed by RNAi when plants were exposed to virulence-causing whiteflies. With the exception of one line that showed a mild symptom, none of the transgenic cotton plants showed indications of normal leaf curl. However, symptoms were evident in the cotton plants that were not transgenic and served as controls. In the Q-PCR analysis of virus-challenged cotton plants, the transgenic T0 and T1 generation plants shown a marked reduction in DNA β satellite, CLCuMB accumulation when contrasted with non-transgenic cotton plants. More specifically, 2,367,884 molecules/µL and 4,015,249 molecules/µL were the amounts maintained by the non-transgenic susceptible control plants, respectively. The CLCuMB concentration was 600 ppm in the T0 producing facility and 15,590 ppm in the T1 generating facility, which is a notable variation.

Correlation of Viral Load and Disease Severity in Transgenic Cotton

Virus abundance was positively correlated with disease severity in T0 and T1 generation plants, according to the researchers. Transgenic tobacco plants have less foliar symptoms when their siRNA sequences match those of the virus AC1, AC2, and AC3 coding regions, according to previous studies. These outcomes are in agreement with those conclusions. G. hirsutum "Coker 310" also showed less severe symptoms when antisense bC1 was expressed. According to the Cotton Leaf Curl Disease severity rating developed by Akhtar and Khan, non-transgenic cotton plants showing severe symptoms of infection, like leaf curling, vein thickening, enation, and stunted growth, are given a score between 4 and 6, whereas transgenic cotton plants showing very mild or no symptoms of infection are given a score between 0 and 1. Consistent with earlier research, this indicates that the severity of symptoms is positively correlated with the concentration of Begomovirus.

To find out how long transgenic resistance from RNA interference to knockdown begomoviral and betasatellite expression at the same time lasts, more research is required. The next step in testing the transgenic plants is to expose them to different strains of CLCuKoV, different amounts of inoculum, and related species of leaf curls that could have sequence similarities with the sites targeted by the viral and betasatellite genomes. It is theoretically possible for a single transgenic construct to provide protection against many viral species and strains, provided that the sequences of the individual betasatellites have been adequately maintained, even though exact sequence homology is not always required. Given the compatibility of leaf curl strains and species with betasatellites, it is expected that a transgenic construct can express both the wild-type activity and an active suppressor of host-mediated post-transcriptional silencing. However, transgenic cotton plants might offer protection against multiple CLCuKoV strains, including the Burewala strain—currently acknowledged as the most frequent begomoviral species infecting cotton in Pakistan—thanks to sequence-specific DNA interference (RNAi).

Gene Expression for Enhanced Pest and Herbicide Resistance

Various studies have documented that overexpression of the respective resistance genes often results in complete control of insect pests or weeds. Using a single herbicide resistance gene (cp4EPSPS) and two Bt genes (Cry1Ac and Cry2A), we previously attempted to change two local cotton cultivars (CRSP-1 and CRSP-2) into a new variety. Although transforming these cotton cultivars was the primary objective, we also compared their gene expression, herbicide tolerance, and insect mortality rate to see how well it worked. Herbicide resistance gene cp4EPSPS and Bt genes Cry1Ac and Cry2A were introduced into plants by the aforementioned shoot apex method. Under identical experimental conditions, the two cotton cultivars' transformation efficiencies differed significantly, largely as a result of their unique genetic compsition.

In terms of transformation efficiency, P CRSP-1 is 1.2% better than CRSP-2, which is 0.7% worse. In terms of Cry2A transformation efficiency, CRSP-2 achieved 0.6% while CRSP-1 achieved 0.9. The transformation efficiencies for CRSP-1 and CRSP-2 were 1.5 and 0.8%,

respectively, for the cp4EPSPS gene. The efficacy of the transformation could be explained by various factors, such as the plant's genotype, the seed embryos' condition, and the genetic composition of the host plant, which influences the locations of transgene integration. Previous research on the efficiency of transformation in different cotton varieties has also shown that the success rate of integration and expression is determined by a close interaction between genetic factors. The two cotton varieties differed noticeably in the expression levels of the Cry1Ac, Cry2A, and cp4EPSPS genes. When tested against identical genes, CRSP-1 and CRSP-2 showed expression at1.2,1.3, and 0.9 ng/ml/unit, respectively. Possible causes of these observed differences in expression include differences in gene insertion sites, plant genetics, T-DNA transfer rates, and Vir gene activity during transformation. The PCR results showed that the transgenes were successfully integrated into both cotton cultivars; it also showed that the Cry1Ac gene, Cry2A gene, and cp4EPSPS gene each had 450, 500, and 350 base pairs of product, respectively. Verification of the effective transformation was achieved by amplifying particular DNA fragments for the associated genes. Results demonstrated that compared to CRSP-2, CRSP-1 expressed more Cry1Ac, Cry2A, and cp4-EPSS. This finding suggests that a wide variety of foreign gene expression potential can be induced by changes in the genetic makeup of host plants.

Four plants of each of the two types and one non-transgenic control plant were selected for the reverse transcription PCR virus titer assay. It's a little unexpected that the plants with the fewest symptoms also had the lowest viral titers. The results of quantitative PCR studies were crucial in the process of choosing plants for the synthesis of T2. Furthermore, the integration of the RNAi gene into T1 plants was confirmed by the subsequent PCR results. To create the RNAi gene, the T2 generation plants were subjected to polymerase chain reaction (PCR) using internal primers specific to each gene. Three of the six MNH-786 plants and five of the eight VH-289 plants from the T3 generation tested positive for the RNAi construct, according to PCR analysis. Using qPCR tests, plants of each variety displaying low and high symptom levels were examined for viral titer. Viral titer levels in both the MNH-786 and VH-289 types were positively correlated with disease index scores, suggesting that the transgenic plants constantly responded differently to virus infection.

The location and quantity of transgene copies in transgenic plants can be detected via FISH analysis. In both cotton kinds, transformants with a single copy were found. Upon closer inspection, it was evident that the transgene locations differed in the two cases. For instance, transgenic plants from line VC2-11 had the gene inserted at chromosome No. 16, whereas transgenic plants from line vv. MC2-8 had it at chromosome No. 6. These findings support previous research that examined the transgenic insertion site for different purposes.

The advent of insecticidal properties tapped from *Bacillus thuringiensis* in the early 20th century gained momentum in finding newer and novel methodologies for pest control. When the Cry proteins are ingested orally, they dissolve and release their toxins over a pH range of 9 to 12. Certain proteolytic enzymes in the midgut of *Lepidopteran* insects initiate the binding of protoxins to transport receptors on the microvilli of columnar cell apical membranes. This specificity confers a peculiar efficiency of the Bt proteins against targeted insect species with the proviso of nontoxicity toward useful insects and other organisms. This property of safety is quite important from an environmental point of view due to the minimal risks for non-target species, including useful entomofauna, birds, fish, and humans. Active research over the years has concentrated on transforming these crystal proteins genes, Bt, into crops especially in cotton as this reduces the application of pesticides which are hazardous to the environment. Crystal proteins of the bacterium *Bacillus thuringiensis* have received widespread attention because of their insecticidal properties.

Comparative Efficacy of Transgene Copy Number on Pest and Herbicide Resistance in Transgenic Cotton Cultivars

Further, copy number of transgenes amongst cultivars was determined by FISH. FISH analysis was then carried out for those plants that exhibited the highest expression of each cultivar's protein. It was determined that CRSP-1 maintained one copy of the transgene, making it more superior in adapting to resistance against insect pests. On the contrary, CRSP-2 contains two copies of the transgene located on chromosomes 6 and 10. These findings agree with earlier studies indicating that single copy number transgenes were highly expressed in cotton plants. To further assess the efficacy of these transgenic cotton lines, insect bioassays were performed along with glyphosate spray

assays. These assessments aimed to measure the resistance of the transgenic plants against insect herbivore and their tolerance to herbicides, thus providing a view on practical applicability of genetic modifications performed. Results of the study have added to the knowledge base in genetic transformation in cotton and constitute part of the basis on which transgenic cotton varieties are being developed that will be tolerant to pests and weeds, hence ultimately improving agricultural productivity and sustainability in the cotton production system.

While the toxicity levels are quite different between the two genetically changed cotton lines, CRSP-1 and CRSP-2, the former has expressed much higher resistance against insect damage in various comparison studies. Quantification of insect damage, in the case of the experimental observations, established that negative controls were damaged between 23% and 90%, in contrast to an impressively very low level of about less than 5% damage caused by CRSP-1. At the same time, the CRSP-2, though with a certain level of resistance, was found to be somewhat susceptible to the attacks by the insects, with the quantified leaf damage estimated at approximately 17%. These findings are in agreement with previous studies that highlighted an increased protective ability of CRSP-1 against pest attacks

Following transformation, the resulting plantlets were selected using the marker genes, and polymerase chain reaction techniques were used to verify that the cp4EPSPS transgene had been successfully integrated. By employing gene-specific primers to amplify a 1.4 kb portion of the cp4EPSPS gene, the effective transformation of the Bt-transgenic cotton lines was further verified. Apart from natural insect resistance features, this integration has in fact given the plants increased resistance to glyphosate. Further rigorous testing was done on transgene expression, using ELISAs to detect the presence of the Bt protein and offer quantifiable information about the transgene's levels of expression in the transgenic lines. Characterization of the cp4EPSPS gene copy number was carried out further and indicated variation among the transformants; one line, CEMB-1317-15, showed a single copy number, while another line, CEMB-1330-4, indicated two copies.

Fluorescence in situ hybridization methods have allowed for the precise visualization of the transgene's insertion inside the plant genome, lending credence to these findings. The absence of signals in

the non-transgenic control plants demonstrated the successful integration of the cp4EPSPS gene. This is consistent with other comparable publications that have employed the same methodology to examine the expression and integration of the inserted gene. A series of bioassays using insects and herbicides were used to test the transgenes' effectiveness; both insect damage and herbicide application were tolerated by these modified plant lines. The strength of the transgenic lines was established by field spray experiments using 1900 ml/acre of glyphosate, which showed encouraging results with potential for commercial application in field circumstances. In the meantime, research on phytochrome signalling pathways contributes significantly to our understanding of how to optimise plant development and yield when compared to the function of PHYB in plants.

Transformation Efficiency and Field Evaluation of RNAi-Modified Cotton Varieties against Whitefly and Viral Attacks

To alter the cotton varieties MNH-786 and VH-289, Agrobacterium strain LBA4404 was used. Eighty-four transformation experiments were conducted in order to transfer the RNAi gene into cotton plants. Approximately 278 from MNH-786 and 196 from VH-289, respectively, showed regeneration after eight weeks, suggesting transformation efficiencies of 3.75% and 2.88%. There have been instances of much greater efficiencies, using Agrobacterium strain LBA4404 on tobacco plants, discovered an efficiency of 20%. Our results supported those of Majeed et al., who discovered that cotton poses particular difficulties throughout the transformation process and recorded a 5.17 percent transformation efficiency.

Subsequently, 43 rooted transgenic plants from VH-289 and 86 prospective transgenic plants from MNH-786 were transferred to pots made of soil. To determine whether the RNAi was present in the cotton genome, we employed polymerase chain reaction, or PCR. Only 24 plants from the T0 generation for MNH-786 and 26 plants from the VH-289 were present when the plants were moved to field conditions during the acclimatization stage. In order to prevent unintentional gene transfer, plants that reached flowering age were let to self-pollinate. To begin the next generation of seeds, ten seeds from each

event were dispersed over the pitch in August 2013. During this time, plants lacked defense systems against whitefly attacks. T1 generation transgenic plants were infested with whitefly soon after sowing and the plants were closely monitored during the course of the attack. The formula developed by Akhtar and Khan was utilized in assessing the viral index for all field plants.

Evaluation of Transformation Efficiency, Protein Expression, and Bioassay Performance

The transformation operation yielded 74 putative transgenic plants after processing 6205 embryos, resulting in a transformation efficiency of 1.19%. This is within the range when compared to studies that show similar crop efficiency using different methods. In conclusion, modern genetic transformation methods show great potential for improving cotton production and alleviating many of the complex issues that farmers face every day. The development of transgenic cultivars with enhanced resistance to pests and weeds will lead to more environmentally friendly farming methods. As a result, chemical inputs can be reduced without sacrificing or diminishing crop yields. Additional study on the potential uses of biotechnology in cotton production lends credence to the idea that agricultural innovation can strengthen food security and economic stability in cotton-dependent countries. Molecular analysis was conducted on all seventy-four putative transgenic cotton plants to verify that the targeted genetic alterations had been appropriately introduced into the plants. The glyphosate-resistant gene, GTG, as well as the Bt genes, Cry1Ac and Cry2A, have been positively amplified in ten of these plants—VH289(2), VH289(18), VH289(25), VH289(52), VH289(53), VH289(55), VH289(66), VH289(69), VH289(72), and VH289(73)—in the T0 generation. The transgenic proteins Cry1Ac and Cry2A, along with the glyphosate-resistant gene product in specific plants, were measured using ELISA in addition to PCR amplification. For the purpose of quantifying Cry2A and GTG proteins, total protein was extracted from each of the ten transgenic cotton lines using standard procedures. Afterwards, specific kits were used to conduct the ELISA.

The highest concentrations of Cry2A and GTG were seen in the transgenic line VH289(18), which had an expression value of 2.79 ng/g. VH289(55) was second with 2.36 ng/g. Expression varied throughout

the transgenic lines, according to the results. The lowest expressions were observed in VH289(69) and VH289(53), with values of 0.44 and 0.74 ng/g, respectively. There was an overwhelming amount of transgenic protein production since ten different cotton plant lines were found to have transgenes in the T0 generation. Their exceptional resistance to insect pests and herbicides was up to ten times more than that of control plants, which was truly astonishing. These findings provide more evidence that transgenic cotton plants containing Bt and glyphosate-resistant genes can be produced more rapidly than conventional breeding methods. This is important from a sustainable agriculture standpoint because it produces plants with resistant traits without putting the ecosystem at risk.

Using the leaf toxicity bioassay, the effectiveness of Cry1Ac and Cry2A, two transgenic proteins, against Heliothis (2nd instar larvae) for extended durations was examined. A leaf bioassay was conducted on young leaves from transgenic cotton plants at 20, 40, 60, and 80 days before crop maturity. For this aim, three larvae were placed into each Petri dish that contained a transgenic plant leaf; the non-transgenic plant leaves served as controls. Researching the toxicity levels of the transgenic protein and maintaining an adequate supply during infestation seasons are of the utmost importance in protecting these crops against *Lepidopteran* pests such as bollworms. Studies have demonstrated that Cry1Ac and Cry2A protein concentrations declined over time. As the toxin levels decline to the lowest range following 0.5-3.0 ng/g, a similar trend is observed in the percentage of insect damage in the T0 putative transgenic cotton plants. The findings are in line with what has been found in earlier studies. Statistics using ANOVA, LSD, and Dunnett's test show that control and transgenic plants have significantly different insect fatality rates. In the entire plant insect bioassay, there was a general variation in leaf damage, which supported previous findings on the transgenic plants' capacity to repel insects.

Furthermore, even though it was suggested that introducing the two Bt genes into the plants would result in insect-resistant cotton plants, the gradual decline in transgenic protein levels is concerning. This can be brought on by the plants reaching senescence or by the promoter region's low activity. The goal of upcoming research should be to produce transgenic Bt proteins in transgenic cotton plant cells in stable concentrations. In addition to evaluating the transgenic cotton plants'

herbicide resistance, a field-based glyphosate herbicide spray assay was carried out on the T0 generation.

Herbicide Resistance Assessment

Following a 45-day observation period, it was noted that three of the transgenic plants displayed necrotic spots, or dead tissues resembling weed plants, and that 10 of the 13 plants that survived and thrived displayed resistance to herbicide. This outcome supports the conclusions of numerous other studies by researchers who have reported that modified crops are resistant to herbicides. Future efforts to develop crops like cotton with greater herbicide tolerance may be impacted by the transgenic cotton plants containing the GTG gene's high levels of glyphosate resistance. FISH was used instead of Southern hybridization analysis to determine the chromosomal locations and transgene copy numbers of the inserted Cry2A and GTG genes in cotton plants. This is a significant strategy since it is well known that transgene copy number and chromosomal location affect expression levels. The analysis's goal, according to the data, was to ascertain the copy number in the transgenic plants exhibiting high levels of Cry2A and GTG protein expression. This supports the finding from prior research that the quantity of transgenes inserted and the variable locations within them have no detrimental effects on the transgenes' levels of expression in cotton plants. As a result, this work has added to our understanding of the transgenic plants' performance by providing insights that are essential to the effective use of biotechnological interventions in cotton crop improvements.

Field Trials

Evidence for this comes from the complete, or near-complete, mortality of insects in field trials and the general health manifested by the growth patterns in this cultivar. Observations like these confirm that the resistance traits have been successfully integrated into CRSP-1 and expressed well, at least regarding weed control. The contrasting performance of the CRSP-2 which, in fact, depicted stunted growth following Roundup™ Glyphosate application at 1900 ml/acre raises critical questions regarding the genetic backgrounds and expression levels of resistance traits in such transgenic lines. It was postulated that the ancestral germ-plasm from which these varieties were derived played an important role in their expression capacities, since one

variety may have just dominantly inherited traits more relevant in resistance to insects and herbicides.

Resistance to herbicides, especially regarding Glyphosate-chemically known as N-(phosphonomethyl) glycine-is a phenomenon that needs an extended discussion. Glyphosate is used to control a broad variety of herbaceous plants, including those with woody stems or branches, as well as those with shorter life cycles (annuals, perennials, and biennials), thanks to its nonselective action. Glyphosate is one of the most important agrochemicals in the world because of its versatility and low toxicity to animals. A key enzyme in aromatic amino acid biosynthesis and involved in various important plant physiological processes, 5-enolpyruvylshikimate-3-phosphate synthase is inhibited by herbicide.

Role of Phytochromes in Plant Growth

The phytochromes are a family of photoreceptors that mediate how plants respond to light. They are involved in a number of physiological activities, including as the regulation of blooming, de-etiolation, and seed germination. To begin with, the 1950s saw the discovery of phytochromes. Among these, PHYB has particular domains that enable it to respond to red and far-red light differently, enabling plants to self-adjust and adapt to their surrounding light conditions. The ability of phytochromes to interconvert between their red and far-red light-absorbing forms enables the plant to sense its competitive environment and adapt its growth further. The growth pattern is highly dependent on the regulating activity of phytochromes, including the establishment of seedlings and the transition from vegetative to reproductive stages. Opportunities for focused genetic intervention to increase agricultural yields were made possible by the identification and characterization of phytochromes.

This present study examines the overexpression of PHYB from Arabidopsis thaliana on cotton plants for variety improvement concerning both physiology and yields. Given the enormous volume of prior work on phytochrome-mediated growth responses in other plant species, we hypothesized that similar improvements could be achieved in cotton through the use of transgenic approaches.

Subsequent molecular analyses revealed that some of the putative transgenic cotton lines indeed resulted in successful integration of the PHYB gene, where out of ninety-nine, seven lines were positive through the use of PCR and Southern blot assays. These lines were further analyzed by using ELISA to assess the expression level of PHYB protein and variable expression was revealed among different transgenic lines. The results from real-time RT-PCR indicated, quantitatively, that line QCC11 exhibited the highest mRNA expression compared to other lines, pointing out the heterogeneity often observed in transgenic plants.

Role of Phytochromes in Plant Light Sensing and Developmental Processes

Phytochromes are a class of photoreceptors found in plants that are essential for controlling the release of chemicals within cells during a number of vital physiological processes, such as flowering and seed germination. They exist in both active and inactive forms, and it has been demonstrated that they function best when exposed to specific red light wavelengths. The light's wavelength determines how these two forms interconvert, illustrating how dynamically plants react to their surroundings in terms of light. Plant pigments called phytochrome serve as the principal photoreceptors for reactions triggered by red and far-red light. The Arabidopsis plant family of phytochromes is PHYA-PHYE, and it consists of five members. Subfamilies PHYB/D/E and PHYA/C have been identified based on amino acid sequence similarity. It has been observed that phytochrome A is more abundant in dark-grown tissues and is light-sensitive, whereas phytochrome B is more abundant in light-grown tissues and is light-stable. The other phytochromes, PHYC, PHYD, and PHYE, are light-stable and perform complex, mostly redundant functions, in contrast to PHYA and PHYB. Phytochrome B is a major photoreceptor that controls factors such as flowering. The main sites for light perception are leaves.

Recent findings have pointed out that this involves the expression of PHYB within the mesophyll cells, not within the vascular bundles, to repress the expression of FT, a major floral inducer in vascular bundles. Overexpression of phytochromes has been tried for several crops. For instance, the genetic transformation of tomato plants with phytochrome A and B genes resulted in higher anthocyanin pigments

and increased rates of photosynthesis. Phytochromes regulate a wide range of processes during vegetative development-from inhibiting stem elongation to promoting leaf development and chlorophyll accumulation-in de-etiolating seedlings. It has been hypothesized that hormones modulate such phytochrome-mediated processes. It is less clear, however, how phytochromes control vegetative development in mature plants.

Scientific studies have proven that plants' ability to respond to light by mediating developmental changes is mediated by intricate transcriptional networks. Shade avoidance, photomorphogenesis in seedlings, germination in seeds, and photoperiod responses are some of these light-regulated activities. For many years, the interest in comprehending the hierarchical structures and composition of the transcriptional networks corresponding to these activities has been fundamental to plant research. To date, traditional genetic and molecular methods have shown to be highly effective in identifying significant regulatory components and their locations within the network.

Light-Regulated Transcriptional Networks and Phytochrome Influence on Growth and Development in Cotton

The transcriptome of plants is extensively reprogrammed by light, as evidenced by recent genomic studies, and transcription factors are much more abundant in early light-responsive genes. When combined, these methods offer fresh perspectives on the transcriptional networks regulated by light. The amount and quality of plant products are directly impacted by the lighting conditions during growth. Numerous investigations on the reactions of photosynthetic photon flux, red (R), far-red (FR), and blue (B) light have been carried out in controlled conditions. Because of the competing absorption and reflection between nearby plants, these wavebands are frequently altered in nature. One of the most popular methods for researching how plants respond to nutritional circumstances and advancing ecological theory is traditional growth analysis. Because it establishes clear connections between physiological and morphological factors, the technique has proven very helpful in ecological research. This allows for a comprehensive interpretation of growth rates and associated characteristics in the context of their environment.

However, due to the limitations of the exponential phase of growth, most physiological investigations stop once reproductive structure has established, and classical growth research has typically only examined the vegetative growth of plants. The ultimate reproductive output of an individual is typically of interest to ecologists and evolutionary biologists, who place more emphasis on the output's fitness than the numerous physiological processes that led to it. To fully understand how plants regulate vegetative and reproductive growth, more data regarding changes in RGR and allocation over the course of the life cycle is required, as plant fitness is dependent on the health of the adult plant and seed output. In cotton, heterologous phytochrome expressions have been reported in recent investigations. This has been followed by assessments of RLRG, LAR, SLW, fresh biomass, and dry biomass for various PHYB-expressing transformants. It is well known that phytochromes control the elongation of fibres, floral induction, and plant shape in cotton. The phytochrome gene family in cotton species has not yet been thoroughly characterized.

This variance in expression levels is most likely caused by factors such as the transgene's copy number, the plant genome's specific insertion location, the promoter type, the inserted gene's nucleotide sequence, and the internal cellular environment. The complex interplay between environmental modulation and genetic components of modification is demonstrated by the fact that this process may be substantially impacted by external environmental influences. From an agronomic perspective, the transgenic cotton lines that overexpress PHYB display distinctive phenotypic traits. Notably, these lines showed an evident extension of time span and photosynthetic rates, along with increased apical dominance and improved leaf morphology. Overexpression of the PHYB gene was recognized to cause increased thickness in the leaves; however, it reduced the overall size of leaves. This phenotype is consistent with earlier studies on phytochromes in which increased photosynthetic capacity was realized in a similar phenotype expressed in transgenic potato plants.

Accordingly, the increased photosynthetic rate in PHYB-overexpressing cultivars could be realized through higher chlorophyll concentration, improved light capture, and photosynthetic efficiency. According to plant physiologists, leaf thickness and chlorophyll content determines photosynthetic efficiency; this consequently would imply that such transgenic variety lines should be adapted to available light.

Physiological measurements revealed that the PHYB-overexpressing lines exhibited considerably higher rates of transpiration, stomatal conductance, and growth metrics in comparison to the control plants. As the primary determinant of plant water use and nutrient delivery, improved stomatal conductance is directly correlated with increased transpiration rate. Since stomata accounts for the majority of water loss, an increase in their width and transpiration rate causes plants to absorb more nutrients and grow more vigorously. In comparison to the control, the PHYB-overexpressing lines exhibited significantly greater relative growth rates and net assimilation rates. Therefore, this association would point to a synergistic impact between higher photosynthesis and improved water usage efficiency, which would boost biomass production. These results are consistent with previous research showing that overexpression of phytochrome genes significantly increased yields in a variety of species. Furthermore, yield measurements showed that the PHYB lines performed roughly 35% better than the control plants, demonstrating once more the potential for major agronomic advantages from targeted genetic modifications in the phytochrome signaling pathways. Beyond just raising yields, the findings have the potential to make cotton production systems more resilient and sustainable in the face of shifting agrarian difficulties. Overall, it is the effective cp4EPSPS gene transformation and expression in cotton, along with PHYB overexpression, warrant additional investigation into transgenic methods for agricultural enhancement. These tactics promote enhanced growth characteristics, yield potential optimization, and resilience to biotic and abiotic stressors. Put another way, combining genetic technologies with traditional breeding techniques is a step in the right direction towards resolving the largest problem facing contemporary agriculture. Even though agricultural practices are always changing, using transgenic crops with improved physiological traits and enhanced resistance traits will be essential to meeting the world's growing food needs. This need must be backed by the application of environmentally friendly agricultural practices. Transgenic technology applications in this regard still require creative research in order to realize their full potential in a variety of agricultural systems. The study's findings demonstrate the connection between transgene expression, physiological function, and agronomic outcomes in transgenic cotton. In addition, by overexpressing phytochromes, these data show significant progress in the integration of resistance traits and the improvement of growth indices. Now is the time to investigate genetic engineering as a viable

option for future crop production increases that are both resilient and sustainable. Maximizing photosynthetic productivity is greatly influenced by a plant's capacity to detect, measure, and respond to various elements of light, including its quality, amount, and direction. Critical developmental processes, like as flowering or entering dormancy, are similarly dependent on a complex system that can detect and react to changes in light wavelength. To adapt to changing light conditions and stay alive in a competitive environment, plants have evolved extremely sophisticated mechanisms. These devices allow us to monitor the changing light levels in our surroundings and adapt our growth and development accordingly. Phytochrome, Cryptochrome, and phototropin are photoreceptor families that send light signals that offer important information about the environment, leading to these adaptations.

Production and Analysis of PHYB Overexpressing Cotton

Given the interest in transgenic approaches, PHYB overexpressing cotton plants were produced. Molecular analyses showed that, out of 100 putative transgenic cotton plants, eight were positive for PCR and further four showed the expression of PHYB as mRNA during northern blot analysis. Interestingly, the mRNA expression level differed, and line QCC11 showed a very high level compared with other PHYB plants.

Compared to other plants with relatively modest expression, QCC11 had an increased mRNA expression, as confirmed by real-time quantitative PCR. Based on these findings, it appears that there is a difference in the expression levels of the various PHYB lines, with QCC2, QCC10, and QCC11 exhibiting significantly higher levels of expression than the others. These results are in line with earlier research that found that different transgenic plants' expression levels were caused by a combination of factors. These factors included variations in the gene's nucleotide sequence, the promoter used, the location of the gene's insertion into the transgenic variety's DNA, the number of transgene copies, and the internal cellular environment.

Transgenic cotton plants that were engineered with Arabidopsis PHYB cDNA under the CaMV 35S promoter provide more evidence that plants overexpressing phytochrome could be useful in agriculture. This report's data should be compared to other plants' data that overexpress PHYB or PHYA. Transgenic cotton plants that

overproduced Arabidopsis PHYB did, in fact, exhibit unique phenotypic traits, such as height dwarfism, enhanced apical dominance, thicker stems, and smaller but thicker leaves. With an extended photosynthetic phase, these crops were also able to experience higher rates of photosynthetic activity. Thus, the dwarf phenotype of cotton plants is quite similar to analogous expressions of phytochromes found in potato plants. Therefore, it would seem that phytochromes indeed play the crucial functions in modulating the time of seasonal germination—a feature that is highly selected against and of great ecological significance. The morphological data, which show an increased trend in photosynthetic rates matching to specific morphological traits, further emphasize the connection between plant morphology and physiology. These results are consistent with those from PHYA overexpression experiments in transgenic tobacco plants. Additionally, the plants that had PHYB overexpressed displayed a higher rate of photosynthesis. The greater chlorophyll content, which results in less excitation of the pigments than in control leaves, is most likely linearly connected to this enhancement. The photoregulation of growth and development of plants depends heavily on phytochromes, which allow them to respond to their light environment.

Agronomic Potential of PHYB Overexpression and the Global Economic Significance of Cotton

Although studies to understand the diverse roles played by phytochromes in different plant species are still ongoing, there is a good probability that the knowledge gained will be put to use to improve agricultural operations. Crop varieties that better satisfy the requirements for growth, productivity, and stress tolerance from the environment may potentially result from ongoing research into the molecular and physiological mechanisms of phytochrome function. Transgenic methods combined with a deeper comprehension of light-responsive signaling pathways may help develop agricultural strategies that are both productive and sustainable in the face of a changing global environment. The transgenic cotton plants' heightened sensitivity to white light caused notable physiological alterations, such as leaf thickening, which in turn led to greater photosynthetic activity. This was demonstrated by the significantly higher production of aerial biomass and the allocation of assimilate to the leaves at the expense of the stems. Compared to other phytochrome B-transformed plants, the transgenic line QCC11 overexpressed phytochrome B with a greater

photosynthetic rate; in contrast, no expression was seen in the control plants. When RLGR and photosynthetic rates for phytochrome B plants were measured, they were discovered to be considerably higher than those of the control group. This is particularly true of the straightforwardly positive association that exists between photosynthetic rate and RLGR. Although quantitative assessments showed that in the phytochrome B plants, fresh and dry biomass ratios were increased by 50% compared to the control plants, this was quite typical for those observations in other works when phytochrome B overexpressed in potato plants provided a higher yield in comparison with their non-transgenic counterparts. The overexpression of Phytochrome B in cotton plants promotes increased biomass allocation to leaves, besides overall health and productivity of plants.

Most tropical and subtropical regions of the world are home to cotton plantations. Known for its dual usage as food and animal feed, this crop is also the top producer in the textile sector, earning it the moniker "multipurpose crop." Cottonseeds are used as a source of protein-rich fodder for cattle and poultry as well as for the extraction of edible oil in many nations. During the manufacturing of textiles, paper, and other items, lint from cotton plants is a major source of higher-quality fibres. Worldwide, cotton is farmed in more than fifty countries, with China, the US, India, Brazil, and Pakistan being the top producers. The globe produces cotton on around 10 million hectares of land annually, with the developing world accounting for 87% of that total. The economic significance of cotton is further highlighted by this, particularly in nations like Pakistan where the crop is frequently referred to as "white gold" because of its vital role in the country's economy.

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Chapter 2: **Maize**

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Economic Importance and Challenges of Maize Production

Maize, after wheat and rice, is one of the most important cereal crops all over the world. In Pakistan, it is being grown on an area of around 1.083 million hectares and hence is playing a crucial part in the economy as well as in food security. This crop is dual purpose, used as staple human food as well as very crucial feed commodity for livestock. Moreover, its usage has also been extended industrially in textiles, food processing, and pharmaceuticals. Maize is composed of 72% starch, 10% protein, 4.8% oil, 9.5% fiber, 3.0% sugar, and 1.7% ash. It has become a versatile ingredient for a wide range of product applications. The nutritional and economic importance of maize justifies the adoption of effective management of pests for maximum production and quality. Improvement in maize productivity involves a multidisciplinary approach, considering improvement in diversified morphological and physiological attributes contributing to growth and productivity. Included are FRW, DRW, FSW, DSW, and root-to-shoot ratios, which will contribute to the full expression of yield potential. Physiological attributes considered include photosynthetic rate, chlorophyll content, transpiration rate, stomatal conductance, and WUE-factors that strongly affect the growth of maize, especially under water-limited conditions.

Maize (*Zea mays L*.) is one of the staple foods that are important to most Africans, as well as an important component in the formulation of feed for livestock. An estimated 800 million people around the world are suffering from hunger, with the greater proportion of this number living in developing countries. Maize has become one of the important crops toward the attainment of the second SDG: Zero Hunger. Currently, the average African yield stands at 1.5 tons per

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hectare, compared with the world average yield of 4.9 tons per hectare. Against the background of this growth in area cultivated for maize, Africa's production amounts to about 7.2% of total world maize output. Various factors contribute to this low rate in production, especially the adverse climatic changes tampering with the quality of maize and food security at large throughout the continent.

Achieving food security in Africa is increasingly being threatened by several interrelated factors that include increasing demand for food, increasing input costs, nutrient degradation in soils, and increasing competition for land and water resources driven by non-food uses. Both abiotic and biotic factors are major constraints to maize yields. Among abiotic factors, temperature is one of the most critical, as it determines the suitable planting dates,, seasonal duration, and general yield. Climatic changes also alter the seasonal precipitation pattern-a factor representing an even higher risk to maize production, since a majority of the farmers in sub-Saharan Africa depend essentially on rainfall for irrigation. Changes in climatic conditions affect not only the availability of water but also the populations of agricultural pests, especially those *Lepidopteran* pests that have acquired notoriety for infesting maize. Five of the economically important stem borer species include the spotted stem borer (*Chilo partellus*), African stem borer (*Busseola fusca*), coastal stem borer (*Chilo orichalcocillielus*), pink stem borer (*Sesamia calamitis*), and sugarcane stem borer (*Eldana saccharina*). Different options have been incorporated into integrated pest management systems to ensure food security in Africa and other parts of the world.

Maize is the third most important cereal crop; it contributes 5.67% towards the total agricultural output in Pakistan with a CCTV area of 1,083,000 hectares and yield of approximately 4,271,000 tons annually. With its dual-purpose nature, maize becomes an important source for human food, feed for the livestock, and raw materials in various industries producing its by-products. Nutritional composition in maize: 72% starch, 10% protein, 4.8% oil, 9.5% fiber, 3.0% sugar, 1.7% ash, 82% endosperm, 12% embryo, 5% bran testa, and 1% tip cap. Crude protein content is high, up to 9.9% during early and full bloom, and it decreases to 7% during the milk stage and further to 6% at maturity, hence its exceptional nutritional value for both humans and animals.

The population of livestock in Pakistan alone is quite high, around 154.7 million heads, which yielded approximately 43.562 million tons of milk, 1.601 million tons of beef, and 0.590 million tons of mutton. Livestock is one of the important sectors within the national economy. It contributes to about 53.2% of agricultural output, according to the record, and 11.4% to the GDP in the country. Green fodder is low input and one of the most important nutritional feeds for the livestock. It contains cellulose, hemicelluloses, fat, crude fiber, acid detergent fiber, neutral detergent fiber, dry matter, ash, carbohydrates, moisture, ether extract, and crude proteins. Production of high quality and nutritious fodder helps to augment the milk production of the animals as high as up to 100%. Volume contribution of fodder crops to the nutritional requirement of the livestock is quite high, standing between 80-90%, but against this total demand, there is a huge shortfall in supply and only 2.46 million hectares of the area are under fodder crop cultivation in Pakistan, thus yielding total production of just 55.06 million tons. This shortage creates severe problems for the nutrition of livestock due to acute deficient months of June-July and December-January. This deficiency in fodder supply is due to a number of causes such as increased human population pressure, erratic and irregular rainfalls, lack of irrigation water, inadequate prioritizing of the production of the crops for fodder, and use of imbalanced fertilizers. With these challenges in view, research efforts ought to be directed toward studying aspects of grain and fodder quality in inbred lines and F1 hybrids of maize. This usually creates the basis for the selection of genotypes by plant breeders, showing better grain output and quality to ensure more food security and agriculture sustainability. Results here indicated significant differences in agronomic traits among maize parents and F1 hybrids, hence indicating a high degree of genetic diversity that could potently be put to use in breeding efforts. The overall protein content from the grains was 9.7396±0.0712%, hence reflecting a great opportunity for protein level improvement by selective breeding. The narrow-sense heritability for this trait worked out to be 96.70%, indicating thereby that it is highly under the genetic control of the respective characters. On the other hand, the lesser magnitude of genetic advance indicates limited expected response through direct selection pressure. Promising hybrids like EV-1097×EV-347 and EV-1097×F-96 recorded better grain protein percentages of 10.77% and 10.67%, respectively, thus serving as potential candidates for further breeding programs for improving protein content in maize.

Maize is the plant belonging to the family Gramineae and known scientifically as *Zea mays L*. It is considered one of the most important cereal food crops worldwide and especially in Pakistan, where, due to a very rapid increase in the population, food supplies have become notably scarce. Maize is considered the third most important cereal crop after wheat and rice in Pakistan. It contributes considerably to the agricultural sector, sharing about 5.67% of the country's GDP. A vast area of 1,083 thousand hectares is under maize sowing, which returns a handsome yield of around 4,271 thousand tons annually. Maize presents various uses; it is directly consumed as food by humans, used as livestock feed, and serves as a raw material industrially in the manufacture of various by-products emanating from maize. The composition of grain itself is about 9.7396% protein, 4.85% oil, 9.4392% crude fiber, 71.966% starch, and 11.77% embryo content. Regarding the silage quality, the Maize consisted of 22.988% of acid detergent fiber, 51.696% of neutral detergent fiber, 28.797% cellulose, 40.178% dry matter, 26.845% crude fiber, 10.353% crude protein and 9.095% moisture content. Despite its importance, maize production in Pakistan is relatively lower as compared to other maize producing countries. The basic reasons for this difference can be ascribed to the lack of access to feasible germplasm as well as resources.

The grain yield of maize is intrinsically associated with other morphological, physiological, and agronomic characteristics; improvement in these traits could be rewarding for improving this crop's production. Genetic correlation assessments provide the basis on which plant breeders can make selections based on strong correlations among the contributing traits of grain yield. It is a vital staple food, while it also plays a huge role in the formulation of livestock feed, therefore being an important crop that could be applied to the support of food security activities. Moreover, maize can be highly valued as a promising renewable source of energy, which increases its value for sustainability in agriculture. However, the best yield is invariably hampered by pest threats from insects that can cause immense losses, among them the maize stem borer, *Chilo partellus*. The control of this insect pest by chemical pesticides has multifarious challenges and disadvantages. Among the major issues encountered, the main attack is generally inside the plant tissue and not within the reach of most chemical applications. This results in farmers resorting very much to heavy dosages of chemical applications, which is likely linked to inducing pesticide-induced

resurgence and outbreaks of secondary pests. The consequences are damage to biodiversity and high risks to environmental and human health. Therefore, there has been an urgent need for developing ecological and economic grounds that are efficient agricultural pest management technology, that is, RNAi technology. RNAi is a common technique used in research to study the function of genes and, more recently, is being adopted as a new approach to the control of agricultural pests.

Maize is the most productive crop in the world due to its importance in countries like Pakistan. Its food production lags far behind population increase. Among cereals, maize is the third most important crop following wheat and rice. About 4.8 % of the total cropped area is occupied by this crop, sharing 3.5 % of the monetary value of agricultural produce. In fact, maize is considered vital in ensuring food security, with a production volume of around 3.34 million tons produced from 0.94 million hectares of land a year. Genetic engineering has emerged as a strong tool for maize trait improvement, especially for herbicide tolerance, through DNA transfer from one organism to another by artificial means within the same or different species.

Of these, the most common methods of plant genetic transformation involve either the gene gun approach, by physically shooting foreign DNA into the plant cell, or utilizing the bacterium *Agrobacterium tumefaciens* that has the ability to naturally transfer its DNA into infected plants. Using this bacterium's method of DNA transfer, the scientists have transferred particular segments of DNA into the plant organism maize and created a genetically modified organism exhibiting desirable traits. Biotechnology is one of the emerging sciences in which Pakistan has gained momentum. Very recently, it joined the list of countries that have commercialized biotech crops. The introduction of Bt cotton through informal channels came as a major breakthrough for the country, and now it is all set to go ahead with the approval of genetically modified (GM) maize for commercial cultivation. Glyphosate is an important part of the agricultural setup: a broad-spectrum systemic herbicide mainly used for weed control. Glyphosate was first patented and sold by Monsanto as Roundup in the 1970s; it saw extensive application after its patent expired in 2000. The mode of action involves the inhibition of enzymes in the synthesis of the aromatic amino acids tryptophan, tyrosine, and phenylalanine. There is

good absorption through foliage, with translocation of glyphosate to the actively growing points of plants. Its activity, however, is confined only to plants in active growth stages. Some of the benefits that have been associated with herbicide-tolerant crops which include: Efficiency in crop weed management, high crop yields, possibility of decreased frequency of application of herbicides, low fuel usage, reduced soil compaction, and the use of low-toxicity active ingredients not persistent in soil. Besides, herbicide-tolerant crops facilitate no-till or conservation-till farming practices that favor better structure and increased soil microbial activity.

Pest and Weed Management in Maize Production

Challenges Insect-pests, pathogens, and abiotic stresses make the production landscape of maize quite challenging on a global scale. The economic losses these threats impart run well into billions of dollars annually in crop loss. Chemical means of control are effective for a short duration but develop resistance among pest populations with time; hence, the need to focus on more environmentally friendly and targeted approaches. Therefore, the methods of control in integrated pest management should involve cultural, biological, and chemical ways of decreasing hazards due to pests. Application of RNAi technology in integrated methods is a very promising way to improve the control of pests with minimum environmental risk. It is possible to reduce dependence on broad-spectrum insecticides using RNAi by targeting essential genes required for survival and development, hence making agriculture more environmentally sustainable. With growing food demands consequent to increasing populations, the challenge of managing pests has become an issue of increasing priority. Through exploiting genetic technologies and RNAi, it should be possible for researchers and practical agriculturists to develop effective methods of protecting key crops such as maize for food security in the future. The inclusion of RNAi technology in pest management practices initiates the start of a new paradigm in agricultural practices through specific target control of pests, further promoting sustainability and conservation of the environment. Further research and development of this technology are essential in overcoming challenges in maize production and improvement of food security at a global level. Genetic Engineering and Biopesticides: The Way Forward in Resolving Challenges Related to Maize Production in Pakistan

The corn leaf aphid, *Rhopalosiphum maidis* (Fitch), is widely distributed and an important pest of maize worldwide. Similar to other phloem-feeding aphids, these pests tap into the vascular system of the plant for access to crucial nutrients using specialized mouthparts known as stylets. This feeding behavior not only deprives the growing maize plant of vital nutrients important for growth and development, but it also inflicts mechanical damage upon the foliar tissues. Other than direct feeding damage, corn leaf aphids transmit plant viruses such as the maize leaf fleck virus that compromise health in plants. Honeydew is produced while aphids feed, and its excretion can accumulate on maize tassels and obstruct pollen shedding, therefore reducing seed set. It is estimated that good control measures against maize aphids could increase maize production by about 14%.

These decisions involve the application of classical breeding techniques and cultural practices to improve the resistance of maize varieties against stem borers while reducing the population of the latter. However, these are time-consuming and lengthy procedures. Chemical pesticides have, till now, been the major means of management of these stem borers in Africa and other developing regions but are habitually associated with immense health and environmental hazards. Moreover, this incorrect application of chemicals has led to the development of increased resistance among the pest populations, and as such, their use is seriously reconsidered. Transgenic maize varieties expressing toxins of *Bacillus thuringiensis* have been developed to impart resistance against these stem borers. However, resistance among the borer populations to Bt toxins has begun to emerge, as well as various negative impacts on non-target beneficial insects. These issues form a serious basis for the need for alternative pest control practices that can effectively be put into agricultural policies in Africa, especially those promoting biotechnology as an effective way to develop stem borer-resistant maize. For this, RNAi technology is employed, which represents an environmentally friendly way of crop improvement through the silencing of certain genes in pests vital for its survival and reproduction.

Climate change exacerbates the situation by impacting the phenological development and productivity of the crop. For example, some evidence has indicated that at the time of pollination, maize productivity is reduced due to extreme temperature events. Additionally, high temperature during grain filling leads to significant

loss in yield; for instance, an estimated fall in maize yield would be about 10% or more with a rise in temperature by 1 °C. Adding all these effects together, insect pests cause severe deterioration to the maize crop. the loss due to the pest accounted for around 59% of the total production loss. Among all the pests that attack maize crops, sucking pests of the order *Homoptera* are the most serious ones. With shifting climate conditions and increased infestation, the problematic stage of these pests will continue to increase. These Bt crops, though engineered with Cry proteins, show negligible resistance to sap-sucking pests, which has caused increased damage to crops. Another factor is that Bt maize shows more susceptibility to aphids, therefore adding to the woes of managing pests effectively. The peculiar feeding attributes of the sucking pests make their control all the more intricate and require novel approaches in an effort to minimize the losses that are going into maize production.

Plants offer their resistance to insect pests due to morphological, biochemical, and molecular responses. These include the synthesis of specific protease inhibitors and entomotoxic proteins such as lectins. The entomotoxic properties of plant lectins, including ASAL, have been widely studied, especially with respect to their activity against insects of the order Hemiptera. For example, in transgenic rice, ASAL expression reduced survival and fecundity of sap-sucking pests. In addition, in several transgenic lines of cotton, ASAL increased resistance to some major sap-sucking insects. In transgenic tobacco, ASAL caused a reduction of about 16–20% in the aphid population. It combined in a unique manner with Cry1Ac to show potent entomotoxicity against the *Lepidopteran* and hemipteran insects. The mode of action of the ASAL gene involved inhibition of nutrient uptake within the insect gut, thereby severely disrupting the epithelial cells. By inducing endocytosis and swelling of epithelial cells, ASAL effectively occludes gut lumen and disables the ability of insects to feed and develop. Further, ASAL binds with a high affinity to various membrane-associated proteins in the gut of aphids, such as sucrase, cytochrome P450, cadherin-like proteins, and polycalins. This interferes with normal metabolism in the gut and subsequently alters feeding behavior in the insect. In the present study, we explored the insect toxicity of the ASAL gene against the corn leaf aphid through in planta bioassays. We generated transgenic maize inbred lines over-expressing the ASAL and further tested their entomotoxic characteristics. Specifically, insect bioassay results demonstrated a statistical increase

in mortality of aphids in ASAL-transgenic maize lines compared to controls. These results obtained in the study indicated that the transgenic lines of maize that expressed ASAL showed a significantly higher resistance to corn leaf aphids compared to untransformed plants. The impressive entomotoxic properties of ASAL on corn leaf aphid point to a great potential of genetically modified crops to highly contribute to sustainability in maize production. The transgenic lines of maize expressing the ASAL gene are highly promising for commercial cultivation and can be used as a genetic resource for developing sucking pest resistance through breeding programmes in future. Furthermore, the ability to cross this transgenic line with Bt lines provides an opportunity to develop hybrid resistant varieties against both chewing and sucking insect pests and elevate the overall tolerance of maize crops to continued climate change and pest infestation. Simultaneously, coupled with the study of genetic variations for resistance to pests, no less attention can be denied to herbicide tolerance during maize cultivation.

Developing ASAL-Expressing Transgenic Maize for Resistance against Sap-Sucking Insects

While Cry toxins have been useful against Lepidoptera and Coleoptera insect pests, they have no effect on sap-sucking insects from the order *Homoptera*. This ineffectiveness is related to the fact that *Homoptera*n insects feed in a special way, which is less dependent on feeding via digestive enzymes and more focused on directly extracting sugars and amino acids from phloem tissues. With these challenges, there exists a dire need to seek alternative and friendly environmentally appropriate pest control strategies that will meet the increasing demands of the human population without adding to the environmental debilitating effects caused by conventional ways of control.

Plants are replete with insecticidal proteins that could easily be developed into an alternative to synthetic pesticides and include lectins and protease inhibitors. Lectins are carbohydrate-binding proteins with high affinity and specificity for various glycans associated with glycoproteins, glycolipids, and polysaccharides. The vital biological functions in the insect gut interfered with by some lectins; for example, those coming from garlic, impede normal growth and development. Several plant lectins have also demonstrated resistance

to *Homopteran* insects, including wheat germ agglutinin WGAb, snowdrop lectin Galanthus nivalis GNA, and concanavalin A ConA. One of the most important ones out of these is insecticidal leaf agglutinin obtained from Allium sativum due to the ability of lectin to bind with glycan structures found in the insect digestive systems.

Transgenic crops expressing Allium sativum leaf lectin (ASAL) have been developed to enhance their resistance against sap-sucking pests. These genetically modified organisms showed an increase in the level of resistance against pests like aphids in various species such as rice, mustard, tobacco, and chickpea. This paper examines the expression of a binary construct that harbors a synthetic ASAL gene introduced into *Agrobacterium tumefaciens* for subsequent transformation into an inbred maize line employed in hybrid seed production. Transgenic maize lines expressed variable levels of the ASAL gene, which were correspondingly variable in resistance against sap-sucking corn leaf aphids.

Genetic Advances in Maize Yield, Quality, and Resistance

The grain oil % also presented a similar trend with an average of 5.27%, and the best hybrid again was B-327×B-316. This is in close agreement with the high heritability estimate obtained as 96.30%, indicating thereby that selection would be effective for improving oil content in the traits studied. However, the modest genetic advance of 8.98% obtained indicates that improvements in oil yields per se are possible but not extensive. The identification of hybrids showing higher grain oil percentages enhances the capability of breeding programs to focus on such lines in an effort to increase production of oil. The crude fiber percentage showed marked variation, as recorded with an average value of 9.4392±0.0579%. Heritability measures were recorded at 91.30%. GA for this character was worked out at 2.66%, showing a moderate possibility of improvement through selective breeding.

Of note, the hybrids E-336×B-316 and E-336×E-322 showed maximum values of crude fiber. That is, such combinations may be promising for further work in connection with developing higher fiber content-which is important for improving the quality of animal feeds. The per cent grain starch, very important for the applicability of maize in food and industrial uses, averaged 71.966±0.1313%. Very high heritability estimate of 91.10% indicates a genetic basis for this trait. However, the

lower genetic advance of 1.25% suggests that restrictive selection practices should be used. Hybrids like B-11×B-316, with as high as 74.20% starch per cent, show promise to improve starch yields. The percentage of embryo, one of the important factors for seed viability, and quality was estimated to be 11.77±0.1120%, reporting heritability as 77.30%. Though the genetic advance was moderate at 2.16%, hybrids like B-11×EV-347 showed an embryo percentage of 12.60%, and hence, out of such, an avenue may be offered to breeding programs for improving seed quality and viability. The quality traits of the fodder were further assessed to identify significant variations in ADF, NDF, cellulose, dry matter, crude fiber, crude protein, moisture, ether extractable fat, nitrogen-free extract, and ash percentages. Because these are important components of maize fodder quality, these various traits will have to be assessed in concert.

The somewhat high heritability values observed across these traits would suggest strong genetic control and would, therefore, be desirable selection targets within breeding programs. Improved values of ADF and NDF in some hybrids show the possibility of improvement in fodder quality, highly essential in livestock nutrition and overall productivity. Generally, these results point to the genetic potentials of the studied maize hybrids for the improvement not only of grain but also of fodder quality traits. The high estimates of heritability that were consistent across most of the studied traits indicate that quite efficient targeted selections could be achieved. Identification of superior hybrids for specific traits has clearly outlined the pathway for future breeding strategies in the optimization of maize for diverse applications in food and feed. Further field testing of the same hybrids is recommended in order to confirm laboratory data and performance across various environmental conditions, which is key to developing stable and high-quality maize varieties.

Current research shows that genetically engineered plastids can generate the necessary quantities of double-stranded RNA-a procedure to control these agricultural pests. Improvement of transplastomic mediated RNAi in African maize breeding programs has great potential to protect livelihoods totally dependent on maize. It may also act as an insurance against impending masculature brought about by stem borer attacks and yield loss under fluctuating climatic conditions. Such a methodology will increase the suite of management options for the African farmer and has the potential to avoid some of

the devastating impacts of both indigenous and invasive stem borers that are almost certain, with time, to develop resistance to conventional pesticide applications. In Pakistan, the most valuable cereal crop is Zea mays, which ranks second in agricultural value after wheat and rice. This fast growth in population outstripped the current food supply of Pakistan. Thus, food security is highly dependent on maize. It contributes about 5.67% to the total value of agricultural output and is grown on area about 1,083 thousand hectares, yielding production of about 4,271 thousand tons annually. Maize has diverse uses, as it is both consumed as human food and used for animal feeding, while it also forms an industrial raw material from which an array of by-products are produced. Nutritionally, maize has a high protein content that reaches 9.9% at early flowering and full bloom.

This is reduced to 7% protein content at the milk stage and further reduced to 6% at maturity. Its grain composition includes 72% starch, 10% protein, 4.8% oil, 9.5% fibre, 3.0% sugar, and 1.7% ash, while in its grain structure, it consists of 82% endosperm, 12% embryo, 5% bran testa, and 1% tip cap. Maize production in Pakistan, though important, is confined to a limited extent as compared to other maize-producing countries due to the limitation of resources and scarcity of potential germplasm. Yield in maize is influenced by a number of morphological, physiological, and agronomic characteristics.

In fact, enhancement of these characteristics can increase considerably the productivity of various maize genotypes. Genotypic correlation analysis owes a greater significance for the plant breeders, who can select the genotypes based on the highest positive association among the traits contributing to grain yield. The heritability and genetic advance analysis across maize genotypes reveal important information about the characteristics that determine yield potential and overall plant performance across ecologies. The magnitude of the heritability estimates, which ranged from 82.35% to 100%, indicated a strong genetic control over the characters and hence proved selection based on such traits will result in meaningful improvement of maize productivity. Notably, the exceptionally high heritability observed for critical traits like photosynthetic rate and chlorophyll content indicates their prime importance, not only for being heritable traits but also as key drivers of growth and yield in maize. The production of maize faces various challenges, and addressing these would unearth new avenues for innovation in breeding practices, biotechnological interventions,

and integrated pest management. This sets the tone for improvements to be made regarding food security in Africa and several other regions whose staple crop is based on maize.

Genetic advance, estimated along with heritability, helps in identifying the correct amount of selection differential to realize desirable genetic gain. Heritable traits identified in the present study with high genetic advance would help in strategizing breeding programs in maize for yield improvement and other adaptive traits. Characters with high heritability, such as fresh root length and photosynthetic efficiency, have been used as dependable selection criteria and can be employed for selecting superior genotypes. Similarly, GCA and SCA are helping breeders to choose genotypes for hybridization providing optimal release in different environments.

This will be a manifestation of the need and importance in determining the various maize parents and F1 hybrid differences in their genetic potentials in the stage. Heritability considers fresh root length and photosynthetic rates, hence availing breeders with an opportunity to make informed selections and enhancing the chances of improving overall maize productivity. Besides, the interaction of different traits and their impact on yield needs to be ascertained. Applying molecular tools and technologies with respect to breeding programs would pace up the identification of favorable alleles, allowing better precision in selection processes.

Pesticides: Usage, Impacts, and Sustainable Alternatives

Pesticides are chemicals used to prevent, destroy, or control pests of plants. They are vital in agriculture and also for the protection of public health. In a broad definition, a pesticide is any chemical or biological agent that is specifically formulated for discouraging, debilitating, or destroying pests. The pests may be broadly grouped into target organisms which include nematodes, pathogens, weeds, insects, fish, mammals, microbes, and birds. Target pests are renowned for causing extensive losses to crops, damages to property, and contributing habitually to the transmission of diseases in humans and other animals, some of which are vector-borne. Application of pesticides, on the other hand, may also affect non-target organisms, sometimes beneficial insects, pollinators, and other wildlife, thus creating relatively large ecological imbalances.

Although pesticide use has the potential to increase crop yields and protect public health by controlling pest populations, there are also associated risks, especially human and environmental toxicity. The Stockholm Convention has listed Organochlorine pesticides as among the most hazardous, toxic, and POPs. Once being widely used as a tool for pest management, such compounds have been nearly banned in most highly industrially developed countries on account of their severe negative environmental and health impacts. The World Health Organization defines pesticides as chemicals intended to kill pests-rodents, fungi, insects, and other plant species that grow where they are not wanted, hence weeds. In application to public health, pesticides have widely been utilized for controlling vector-borne diseases such as malaria and dengue fever.

They help in agriculture by repelling threats to crops that may significantly hinder crop production, thus affecting national food security. A great deal of care and safety measures must be taken when handling pesticides, mixing, or applying them in order to minimize risks from human exposure, especially when accidents occur in improper disposal or contamination. Pesticide is further described by the Food and Agriculture Organization as any substance or mixture of substances intended for controlling, preventing, or destroying pests, including vectors of human or animal diseases and unwanted species of plants or animals. This definition outlined the multi-dimensional nature of pesticide use in every aspect of agricultural production, processing, storage, transport, and marketing of food and animal feed. Some pesticides are, further, formulated for use as plant growth regulators, defoliants, desiccants, or active agents that prevent preharvest drop of fruit, to show various manners of use of the chemical active agents in agronomic practice.

The global consumption of pesticides is approximately 2.5 million tons annually, with a steady increase observed over the years. Similarly, in Pakistan too, the situation pertaining to the use of pesticides also has serious public concerns. In developing countries, around 37,000 new cancer cases annually are directly related to pesticide exposure. Recent reports indicate that nearly three million are engaged in various types of pesticides, such as 39 types of weedicides, 5 types of acaricides, 30 types of fungicides, and 6 different types of rodenticides. Due to the ever-increasing trend of imports related to pesticides, the chemicals are becoming indispensable and have reached to about 80% of the

total application on cotton crops of the country. According to the World Health Organization, it is approximated that about 200,000 people die every year from pesticide-related diseases all over the world, and a big proportion of the total number of cases comes from developing countries. In Pakistan, cotton apart, even tobacco, sugarcane, paddy, fruit of different varieties and maize are all subjected to applications of pesticides. According to the Technical Bulletin, pesticide use in the country has increased by 1169% in the last two decades. Even a single crop is sprayed more than ten times. These practices thus pose a potential health risk for humans and a question of environmental integrity.

The misuse of pesticides is further aggravated by the low literacy rates among farming communities, as most pesticide labels are either in English or Urdu. This implies that more than 70% of farmers lack an understanding of how to handle such chemicals safely, hence applying them incorrectly and increasing their health risks. Most female workers are involved in cotton picking and hence are likely to face high exposure to pesticides. Field research on the cotton-growing areas of the country showed that women workers showed more health problems from pesticide application than men. Accordingly, headaches were found to occur with 26%, gastroenteritis by 10%, skin irritation by 27%, and nausea by 12% of the surveyed female workers. Such health maladies may be worse if the women frequently work near cotton fields since some varieties were grossly sprayed with pesticides. Atmospheric transport of POPs has been recognized as an important environmental issue, accounting for 80-99% of the total reservoirs in the world's oceans. However, very little work has been carried out so far in Pakistan to address the extent of pesticide contamination in the atmosphere, whereas considerable quantities of these chemicals are used in localized agricultural practices.

This generally results in lessening of the populations of necessary soil microorganisms that apply with heavy use of these chemicals. According to Dr. Elaine Ingham, one of the world's leading soil scientists, there is no way the health of the soils can ever improve where key microbial communities, like fungi and bacteria, are compromised. Massive overdosing with chemical pesticides associated with chemical fertilizers can severely disrupt the dynamics of the soil microbial ecosystem, much as human health can be devastated by overdosing on antibiotics. Though the initial effects appear to be

promising, the continued usage of pesticides leads to resistance in the soil with a consequence of inefficiency in nutrient cycling by the microorganisms. Degradation of such type reduces the capacity of soil to fix atmospheric nitrogen into accessible forms for the plants. The exposure to pesticides from human beings results in a wide range of health manifestations of both acute and chronic types. The spectrum of serious health outcomes due to pesticide exposure includes disorders that vary from simple irritation to life-threatening health conditions. Common manifestations include irritations of the skin and eyes, rashes, and respiratory complaints. However, in chronic toxicity, more serious health issues like neurotoxicity, hormonal imbalances, and a variety of cancers have also been reportedly linked to it. Epidemiological investigations have documented an association between pesticide exposure and various cancers, mainly leukemia and also non-Hodgkin lymphoma. What is observed creates a strong impetus for the need to reduce pesticide use to decrease the health effects of pesticides. Organophosphate insecticides document mounting evidence of neurobehavioral changes in addition to other illnesses like death, birth defects, and neurological impairment.

Exposure to pesticides has differentially reported a number of cancers among the people of Pakistan, including breast, ovarian, prostate, and testicular cancers in both men and women, which indicates more crucial public health importance. The rising magnitude of these health issues requires more effective and scientifically plausible preventive measures in order to avoid the public health crisis, environmental deterioration, and soil contamination due to heavy and indiscriminate use of pesticides. The agricultural sector saw a steep rise in the production of pesticides along with the development of farming practices. Pesticides have become so important to many farmers that, besides just crop protection from pest attacks, they also facilitate higher yields, stimulating increased production and attracting many companies to develop their proprietary formulation. This has integrated pesticides into daily agricultural practices, and every year, farmers continue to widely implement new chemical agents for crop protection. The aftermath or the repercussions are of an adverse nature, as it helps in skyrocketing food prices along with environmental damage. In such a backdrop of challenges, some novel approaches have become imperative for which development of GMOs could be an alternative to reduce the over-dependence on pesticides. Through such biotechnological interventions, the resilience of crops against

certain pests and diseases could be improved and thereby decrease the usage of chemicals. Whichever be the strategy or approach, it is very important to increase food production in a way that ensures quality and safety of food and controls pesticide residues for protection of biotic life and reduces environmental deterioration. Whereas there are benefits that come along with the use of pesticides, significant issues concerning its application on crops have to be considered.

The public health significance of pesticide use came into sharp focus during the outbreak of the dengue virus in October 2006, which claimed 44 reported deaths. In a drive to control this vector-borne disease, the government implemented general spraying of deltamethrin in highly urbanized areas. This intervention was poorly executed without proper precautionary measures being taken, and respiratory complications started to rise among the populace. The hospital records showed an over 20% increase in respiratory problems during this period, and environmental conditions in cities like Faisalabad and Lahore had misty atmospheres, with a temperature ranging from 13 to 20°C. These conditions act in favor of persistence of the pyrethroid applications, hence increasing exposure risk to its residents. The use of a number of different chemicals in the agriculture sector in Pakistan, joined with their application on major crops such as sugarcane, maize, oilseeds, fruits, vegetables, and wheat, has resulted in generalized environmental pollution spreading throughout most of the provinces in the country. Though pesticides have considerable value in sectors such as agriculture, forestry, domestic applications, and public health, even in the face of actual losses of human life, the risks these chemicals/pesticides create to human and ecological health are significant.

The improvement in yields both in livestock and crops arising from the effective control of pests by application of pesticides is recorded, and thereby contributes to improved food security. Nevertheless, this widespread application of pesticides brings into view some questions regarding possible undermining potential, especially in developing regions where regulatory mechanisms may be ineffective. Depending on use, pesticides undergo several fates in the environment, including photochemical transformation leading to the production of harmful or less harmful and less toxic metabolites to human beings and the environment. Besides uses in agriculture, the transport sector has also

increasingly utilized herbicides and insecticides for maintaining grass pastures in sports pitches, golf courses, and playgrounds. Besides, pesticides are important in the protection of buildings and other wood structures from destroys caused by termites and other insects that destroy wood.

In the discipline of public health, pesticides are best known for their incredible value in the control of diseases carried by vectors, which are responsible for tremendous mortality and morbidity. The insecticides are a very important tool in preventing the spread of fatal diseases like malaria and dengue. deaths reported due to malaria have been estimated at about 5,000 daily in India alone. Malaria still accounts for much mortality and morbidity in developing countries. The public health effects of pesticide use create an imperative for comprehensive programs that manage pest populations with minimal human health risks. Though invaluable in this regard, little can gainsay the fact that pesticides indeed help in augmenting agricultural productivity by bringing about reduced losses from weeds, diseases, insects, and pests of crops. While pesticides serve to enhance the continuing improvement of food quality and agricultural outputs, their safe and responsible use is crucial in safeguarding human health and environmental sustainability. The problems posed by pesticide exposure-most especially in developing nations-call for an increase in education and awareness among agricultural workers, a more rigid regulatory framework, and encouragement toward integrated pest management practices that value ecological health alongside human safety. In addressing these problems, balancing between effective control of the pest and the need to protect the environment, as well as public health from adverse effects associated with pesticide use, becomes paramount. Pesticides on the one hand have contributed to crop production through the control of pests, but on the other hand also bring along very many adverse effects on human health and the environment. Their improper use results in pollution of the primary vital resources like water, soil, meadows, and vegetation. Along with their target of destroying the injurious insect population, they are also toxic to non-target organisms such as beneficial insects, birds, and plants. Of the classes of pesticides, insecticides are generally considered to be among the most toxic; however, herbicides have frequently been cited as being disproportionately hazardous due to their broad range of toxicity across non-target organisms. The use of

pesticides in agricultural practices contributes negatively to the soil ecosystem.

The term "pesticide" encompasses a multidiversity of chemical classes including herbicides, molluscicides, fungicides, nematicides, rodenticides, and plant growth regulators. Of these, the Organochlorine pesticides attained widest application due to their great effectiveness on a wide range of diseases, from typhus to malaria. However, their use has been much curtailed since the 1960's, mainly because of their persistence in the environment and health risks. This has dramatically altered the approach for managing pests with the discovery of other synthetic insecticides such as pyrethroids in the 1980s and organophosphates and carbamates in the 1960s and 1970s, respectively. During their time of application, these molecules played a major role in combating pests and agricultural output. For that matter, pesticide design is to be lethal to only the target organism while it is ideally quite harmless to other non-target ones, including humans. However, overuse brought into effect some serious ecological impacts and the development of controversies regarding misuse and over-dependency on these chemicals. The class of pyrethroid insecticides is one of the synthetic pesticides, which have been under much attention owing to their very fast insecticidal action emanating from pyrethrins, natural compounds extracted from chrysanthemum flowers.

While pyrethrins are effective, it is important to consider that the natural constituents of pyrethrins are not always lethal and degradation will have long-lasting effects on the environment. Pyrethroids have been chemically tailored to improve factors such as persistence in agricultural uses and higher toxicity for longer-term effectiveness against target pests. While this extended efficacy is desirable for the management of pests, it also raises several concerns about environmental contamination and the development of resistance in the pest population. This has brought into focus the increasing awareness of the need for integrated pest management strategies involving biological control methods, cultural practices, and judicious use of pesticides that minimize environmental impact while maintaining agricultural productivity. Though important to note, pesticide toxicity represents a direct and acute poisoning of target pests; however, the potential risks of pesticide use include but are not limited to an outcome such as this. More serious consequences involve

long-term injury from pesticide residues in the soil, water, and food supply chain. The likelihood of bioaccumulation among non-target organisms like useful insects and wildlife, particularly, places a premium on the need for environmentally-friendly management of pests. Moreover, pesticide resistance among pest populations indicates the need for further research and increase innovation in pest management approaches.

Advanced Pest Control and Sustainable Approaches

Over the last ten years, the trend has changed to seeking alternative methods of controlling pests that are viable as well as environmentally friendly. Being derived from natural organisms or substances, biopesticides hold great promise in the quest to reduce reliance on synthetic chemicals. Options like these offer very effective control of pests while ensuring no or minimal adverse impacts on non-target species and the environment. Moreover, the precision in agriculture technologies allows for more selective pesticide applications, reducing the overall use of chemicals and increasing efficiency in pest management.

Awareness among the general public regarding pesticide use and the regulatory mechanisms in the light of the issues raised by these substances continuously evolve. Stricter controls and directives regarding the handling and application practices, in general, and waste disposal procedures for pesticides aim at taking greater care towards human health and environmental issues. Further, sensitization of farmers and agricultural professionals concerning the potential dangers involved in pesticide applications and encouraging methods of sustainable agriculture might ensure the responsible approach towards pest control. Long-term effects of pesticides on ecosystems, human health, and food security are active areas of research toward the development of comprehensive strategies that mitigate risks but maximize agricultural productivity. Further efforts will be needed from scientists, policymakers, and agricultural stakeholders in shaping this future toward practices of sustainability in pest management. The impact of pesticides on biodiversity forms one of the critical areas of concern, since loss among useful insects, pollinators, and other non-target organisms can have cascading effects on ecosystems. Their interconnectedness in agricultural landscapes signals the need for

holistic approaches-that take into consideration wide ecological implications of pesticide use. Publicized biodiversity and facilitated beneficial organisms by farming practices improve the natural pest control mechanism, hence reducing reliance on chemical intervention. In the ever-evolving face of agriculture, traditional knowledge allied with modern science and new technologies will play an important role in shaping farming systems resilient not only to pests but also to environmental changes. Sustainability-oriented pest management can offer a healthier environment and more secure food supplies in order to help tackle some of the key challenges of food security and ecological health in this rapidly changing world. The complex interplay of pesticides, agriculture, and environmental health underlines responsible pest management. Having a sustainable practice in the agricultural industry that takes into consideration ecological consequences from pesticide use will balance productivity and stewardship of the environment. The further development of integrated pest management practices will continue to be integral in combination with advances in agricultural science to meet the immediate and long-term needs of food production systems in such a way as to protect human health and the environmental base upon which life depends. Pyrethroids are frequently applied in tank mixes or premixed formulations with other insecticides to enhance efficacy on target pests. Of the better-documented poisoning symptoms associated with pyrethroids, honeybees that have been exposed will regurgitate nectar after collection. Incidents like that show the risk these chemicals can impose on the target pest population and even on beneficial insect species, which are very vital in effecting pollination and maintaining overall ecosystem health.

Pyrethroids could be categorized into two classes, including Group 1 and Group 2, whose efficacy is based on active ingredients. Bifenthrin can be described to be one of the most common pyrethroid insecticides; its chemical formula is $C_{23}H_{22}ClF_3O_2$. This chemical is mainly used in the control of the red imported fire ant by acting on the nervous system of the insect of concern. Bifenthrin is highly toxic to aquatic life, thus posing an environmental hazard. However, regulatory authorities have allowed the sale of bifenthrin for common usage, provided that the concentration levels of bifenthrin in the commercial formulations that reach the end user remain low. Bifenthrin is classified as a restricted-use pesticide in the United States due to its hazards.

The development name of bifenthrin is FMC 54800, and the commercialization was started by the FMC Corporation. It is present in many products, including Ortho Home Defense Max, Transport, Brigade, Zipak, Mega Wash, Scotts Lawn Pro Step 3, Maxxthor, Wisdom TC Flowable, Bifen L/P, Allectus, Bifen XTS, Ortho Max Pro, OMS3024, Talstar, Bifen IT, Capture, and Torant. Such a wide range of products underlines the extremely widespread dependence on bifenthrin within pest management strategies. Long-term experiments concerning bifenthrin toxicity have been conducted on different types of animals. An extended experiment on male and female Swiss mice at ages of 20 to 21 months, for example, showed increased incidences of urinary bladder tumor in males when compared in a pairwise manner. Similarly, in females, indications of elevated risks existed for lymphoblastic leukemia and lung tumors under similar comparative conditions. In contrast, another feeding study conducted into Sprague Dawley rats of both male and female gender for a period of 104 days showed no significant evidence of tumor development that would need medical intervention. The scenario in India as far as pesticide production is concerned has changed a lot since the setting up of the insecticide manufacturing plant near Calcutta in 1952. At present, it is the second largest pesticide producer in Asia, next only to China, and occupies the twelfth rank in the world for production.

The manufacturing capacity of mechanical-grade insecticides in India has rightly gone up from 5,000 metric tons in the year 1958 to over 102,240 metric tons by the year 1998. In the financial year 1996-97, the market value of pesticides in India stood at an estimated at about Rs. 22 billion or about USD 0.5 billion and accounted for nearly 2% of the total world pesticide market. The pattern of pesticide usage in India also differs significantly from the global pattern. Whereas 46% of the world pesticides are used as insecticides, the percentage increases phenomenally to 76% in India. Further, around 45% of the total pesticides applied domestically are reserved only for cotton varieties, followed by consumption in rice and wheat cultivation. This heavy reliance is essentially centered on the dire need for pest control among the major cash crops, which are quintessential for an agricultural economy.

It is important to understand the level of toxicity and severity of risks likely to be caused to both biotic and abiotic components of the environment, as elaborate documentation on adverse impacts due to

pesticides to human health and ecosystems is well documented. The present study was therefore undertaken as a critical biosafety study to investigate the effects of pesticide use on maize crops using broiler chickens and Wistar rats as model organisms. Maize crops were cultivated in an environment specifically sought for the right observation of pesticide effects. In this experimental design, there were four distinct broiler chicken groups: one group that did not have pesticide poisoning, one normally poisoned with pesticides, one poisoned with high dosage pesticides, and one poisoned with low dosage pesticides. Whilst carrying this out, the rats employed in the experiment were the Wistar rats that were divided into two groups: the control group and an experimental group exposed to pesticides. Soil analysis was performed both before and after the maize harvesting process by considering nine parameters that would give the impact of pesticide application on them. Some of these parameters are sodium, potassium, phosphorus, electrical conductivity, pH, and soil texture, among others, giving a picture of the overall health of the soil ecosystem. Notably, it was observed that no significant changes in the quality of soil existed subsequent to the application of pesticides.

Recordings of weight were very carefully taken among chickens at four stages in the entire 45-day trial period. Data analysis was done using Excel 2013, which approves the graph representation of weight variation between the experimental groups studied. It can be observed that during the period of study, all the groups into which the chickens were placed-that is, control, normal, and low-dose pesticide groups-increased in weights. A highly significant reduction in weight was observed at the end of the trial in the high-dose pesticide group. Similar trends have been observed in the control Wistar rats, which showed increased body weight, whereas many experimental rats exhibited a decrease in weight, both further indicating the possible effects of pesticide exposure. The organ weights of the various collected organs of chickens and rats were measured through post-mortem examinations. Results have shown that the organ weights of the segmented organs fell within the normal range, and no marked differences were observed as compared to the control group.

RNAi and Biotechnological Strategies in Maize

While this concept is promising, the application of RNAi in the management of insect pests is complicated due to several factors, which includes poor delivery mechanisms, variable RNAi efficacy, and degradation of double-stranded RNA.The main problem with widespread use in agriculture is identifying appropriate genes that can be targeted for pest control. However, reports of RNAi application have been rather successful across different insect orders, although limited success has been reported in Lepidoptera species. This analysis has sought to apply HIGS by targeting the chitinase gene as a potential target in host-induced gene silence. Chitinases belong to the O-glycoside hydrolase superfamily and catalyze the hydrolysis of chitin, yielding oligomers of N-acetylglucosamine. They participate, amongst others, in moulting and digestion since they allow degradation of chitin from the exoskeleton and from the peritrophic membrane of insects.

Although the genes that encode chitinases have been identified and characterized in several *Lepidopteran* species, such information is still lacking in *Chilo partellus*. Suppression of chitinases and chitin synthase expressed in previous studies has resulted in major changes in the development of many insect species, whichFlags their importance as potential targets for the control of insect pests. This research focuses on the identification and determination of chitinase genes in *C. partellus* and their expression profiles during various developmental stages. More so, we tested the effectiveness of chitinase dsRNA in inducing the RNAi response in *C. partellus* by comparing the RNAi knockdown using bacterially expressed dsCHI and purified dsCHI. Further, in the study, we sought to provide proof of the principle of RNAi-mediated insect control via transgenic maize plants expressing dsCHI targeting chitinase in *C. partellus* . Application of RNAi technology is, therefore, considered a potential way to control insect pests without affecting other beneficial insects in the ecosystem, since it is highly specific. Thus, targeting genes that are vital and have potential to cause harmful effects on the target insects, along with efficient mechanisms of dsRNA delivery, are other important factors contributing to effective RNAi responses. Given that *C. partellus* is an invasive pest with great economic impact due to its considerable damage to crops of both maize and sorghum, this present study is aimed at establishing the efficacy of RNAi against this target by

targeting chitinases specific to this pest, thereby opening more ways to further improve the strategies of pest management.

Chitinase: mechanisms, applications, and control strategies in agricultural insect pests

Chitinase enzymes from the glycosyl hydrolase family catalyze a very important process of chitin digestion, which is a structural polysaccharide of insect exoskeletons and the peritrophic matrix lining the midgut of most arthropods. Enzymatic activity of chitinases facilitates the biological processes of molting and digestion, and hence influences growth and development in insect pests. Thus, the payback of new pest control strategies has also pinpointed these enzymes as targets, particularly in economically important crops like maize.

These range from different delivery modes of dsRNA to insects, like oral feeding, microinjection, soaking, and transfection. Of these, one of the most promising approaches involves a method known as host-induced gene silencing, in which transgenic plants are employed that constitutively express dsRNA. This technology allows for the selective knockdown of genes involved in reducing insect pest populations that could minimize crop damage. Since RNAi is a very highly specific technique, HIGS would be more environmentally friendly than conventional chemical pesticides, which often possess broad-spectrum activities and may affect the beneficial insect populations. Here, we focus on the oral delivery of bacterially expressed dsRNA targeting chitinase genes in the Spotted Stem Borer, *Chilo partellus*, a noxious insect pest considered one of the most economically important insects affecting maize crops in Africa and causing yields to be considerably lowered each year. In this paper, we explore the efficiency of dsRNA in inducing gene silencing and the eventual consequence of such silencing on larval survival and growth.

Previous studies have shown that feeding-based RNAi has the potential to trigger an effective RNAi response in agricultural pests. Normally, the efficiency of RNAi depends upon the availability of dsRNA in the insect gut in a sufficient quantity to initiate the silencing pathway. However, in practice, it is often difficult to achieve this concentration, especially when naked dsRNA is used, which normally has very low stability in the insect gut. The results indicated that larvae treated with

bacterially expressed dsRNA had a significantly reduced chitinase gene expression, corresponding to their mortality rate. On the contrary, the larvae that received pure naked dsRNA seemed less sensitive, probably because of the rapid degradation within the gut. Degradation of dsRNA in the gut takes place because there are ribonucleases that deteriorate its stability-hence, it would be important to protect it from such interference if one wants to increase the success of RNAi.

Recent reports have indicated that different insects possess variable amounts of dsRNA degrading enzymes which may drastically affect the RNAi effect. Our results indicate that the longer the larvae of *C. partellus* are subjected to bacterially expressed dsRNA, the stronger the silencing effect becomes - consistent with previous studies. This is because the naked dsRNA degrades rapidly and for this reason is unable to effectively silence target genes. This is further exacerbated by the competitive interaction between dsRNA and ribonucleases, which may reduce intact dsRNA available for uptake by the RNAi machinery. The dsCHI expression in transgenic maize lines was done using a constitutive promoter, the CaMV 35S promoter, along with the enhancer of the Ubiquitin promoter. These plants expressed the dsRNA at variable expression levels, which could be a result of different integration sites or copy numbers of the gene present in the genome.

Subsequent behavioral bioassays revealed that *C. partellus* larvae preferred non-transgenic leaves to those of the transgenic maize, indicating a potential role of the transgenic lines in feeding deterrence. Feeding behavior becomes quite important in this aspect: challenging the larvae with the transgenic leaves highly reduced Chitinase transcript levels and resulted in very high mortality among the larvae, showing the effectiveness of RNAi against the expression of essential developmental genes.

Genetic Engineering for Pest and Herbicide Resistance in Maize

Although it is rare, the genetic basis for weed resistance to glyphosate has been reported to involve two major mechanisms: one is enhanced detoxification capability and the other an altered biochemical site of interaction with the herbicide. Both mechanisms bring modifications either to protein functioning or expression. In the case of

detoxification, enzymes that facilitate the breakdown of glyphosate are produced in greater quantity or possess enhanced activity. Biochemical interaction site changes: These are changes in enzymes or receptors so that their affinity for glyphosate is much weaker compared to conventional ones. Glyphosate-resistant crops are, therefore, one of the histories in change in agricultural technology as far as sustainability of farming is concerned and increased crop yields.. This is genetic engineering for resistance to pests and tolerance to herbicides-the integrated approach to solving some of the major issues in maize production. The employment of transgenic technologies for developing crops resistant to sap-sucking pests and tolerant of herbicides will have the dual benefits of optimizing maize yields with minimal environmental impacts and reliance on chemical pest control methods. The combination of these traits holds the potential to develop hybrid varieties, offering a way toward resilient agricultural systems-ones that are conducive to food security for growing populations, particularly in regions like Pakistan where there is an immense challenge in agriculture.

Genetic engineering with the use of Agrobacterium-mediated transformation enables developing glyphosate-resistant maize varieties. The main objective is usually to establish a competent transformation system and produce stably transformed maize inbred lines which are glyphosate tolerant, hence facilitating the development of hybrids and synthetic varieties with improved grain and fodder yield potential. Weeds compete with the most economically important crop, such as maize and sugarcane, for resources and interfere in harvesting. Glyphosate-resistant maize provides a solution to increase the ability to manage weeds with post-emergence applications. This obviously creates greater flexibility in managing problematic weed species and can have significant economic advantages for growers. The glyphosate-tolerant gene was then cloned into the pCAMBIA-1301 vector, which is derived from the pPZP vector family. This vector has been designed to support high copy numbers in E. coli for increased DNA yields and to maintain high stability within Agrobacterium. Its compact size, with carefully chosen restriction sites to enable modular manipulations of plasmids and poly-linkers, allows translational fusions to GUS reporter genes. The plant expression vectors contain the plant selectable marker genes, hygromycin and GUS, as well as the glyphosate tolerant gene, all driven by the CaMV35S promoter, terminated by the CaMV35S Poly-A signal. This cassette has commonly been used in

several transformation protocols of maize because it allows constitutive expression of genes throughout an entire transgenic plant.

Thus, the gene tolerant of glyphosate was positioned between the NcoI and BglII restriction sites. This vector was previously used in transformation studies of numerous plant species and has been used in several rice transformation studies. To test the efficiency of transformation, 2,000 embryos were isolated from each of the inbred lines CIL-123 and CIL-194. After transformation, a total of 41 transgenic plants were obtained from CIL-123 and 48 transgenic plants from CIL-194 after selection for 4 to 5 weeks with 50 mm glyphosate. It was also mentioned that glyphosate is an efficient selective agent, but when supplemented in media, it prevents root development; on the other hand, an optimal concentration of glyphosate has to be maintained in order not to get false positives due to other selective agents such as kanamycin.

GUS assays were also conducted to determine the expression of the gene in the transformed plants. These confirmed the active presence of the GUS reporter gene in the actively growing young leaf tips, showing fairly uniform patterns from GUS-positive plants. The β-glucuronidase gene is generally known to act as one of the efficient reporter genes among a wide range of plant species. When integrated in the plant genome, the GUS gene, followed by incubation in GUS substrates, produces marked blue coloration in plants. Hence, it provides a dependable marker for gene expression. Transformation in plants using GUS as the reporter gene has been reported earlier. The histochemical GUS assays conducted on the transformed com leaves showed blue spots indicative of successful transformation due to the presence of the glyphosate-tolerant gene. Non-transformed leaves didn't show any coloration. The appearance of the blue color on the leaves showed that a huge amount of GUS protein was expressed in the two transgenic lines confirming the presence of the Glyphosate tolerant gene. Total crude protein content was measured by the Bradford assay in GUS-positive plants, while levels of expression of specific target protein were measured by ELISA. Putative transformants selected on the glyphosate medium were analyzed by PCR using specific primers. An ~1.4 kb long PCR fragment confirmed the presence of glyphosate-tolerant gene in positive maize plants. With verification of transformed plants by PCR, the genes expression was further detected using Dipstick assays and ELISA techniques, hence

establishing the expression of the respective proteins. This ELISA technique, relating to the antigen-antibody relationship, is among the most utilized techniques for detecting specific protein expression in transgenic plants.

While both the Dipstick assays and the ELISA gave positive results, it was an indication that the plants had been stably transformed and were actively expressing the glyphosate tolerant gene. After verifying that this indeed represented a successful transformation, those plants that gave positive results were selected for further analysis and field trials. Meanwhile, two lines of maize were examined in this current investigation concerning the gene for glyphosate tolerance, namely: CIL-123 and CIL-194. The results showed that the line CIL-194 surpassed the performance of CIL-123. Field testing was done in order to predict the potential of the lines in terms of grain yield. Higher heritability in the transgenic lines showed that selection for the respective trait could allow the production of hybrids that possess improved yield and quality characteristics.

In addition, the study identified key agronomic traits that were significantly associated with the improvement of grain yield to include: stomatal conductance, photosynthetic rate, number of cobs per plant, cob length, cob diameter, chlorophyll content, number of grain rows per cob, grain protein content, grain oil content, grain starch, embryo percentage, and moisture percentage. High heritability recorded for these traits justifies the effectiveness of selection for bringing improvement in crop production and for the optimization of genotype selection for breeding purposes. The application of correlation and regression analyses further proved the role of certain traits in enhancing the productivity of crops. Principal component and factor analyses were conducted in order to develop selection criteria using the large number of traits that were evaluated through the duration of the study. These different analytic approaches would complement very well the identification of the most promising traits that ought to be focused on during the breeding efforts. In essence, we highlighted the importance of the development of glyphosate-tolerant maize varieties using genetic engineering techniques and has indicated that such transformed lines could contribute to agricultural productivity while minimizing some of the challenges related to weed management in maize cultivation.

Exploration of Future Control Strategies

Our study consequently supports RNAi-based control as a viable alternative to the traditional use of chemical pesticides. Because RNAi is highly specific, targeted gene silencing with minimal unintended impacts on non-target organisms and the environment is achievable. Keeping in view the drawbacks of naked dsRNA, various researchers have started exploring nanoparticle-based delivery systems that protect dsRNA from degradation and enhance its stability and bioavailability. So far, nanoparticles have shown promise in protecting dsRNA from ribonucleases while delivering it effectively into insect cells. This novel methodology can increase the efficacy of RNAi in controlling pests and could be one of the most sustainable and environmentally friendly concepts for crop protection.

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Chapter 3: **Potato**

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Introduction to Potato Viruses and the Impact on Agriculture

Genome sizes of potato virus X vary from 5.9 to 7.0 kilobases, and the virus is a single-stranded positive RNA. It is considered to be a member of the genus *Potexvirus*. One of the most prevalent viral infectious agents that infect potato crops and spread mechanically is PVX. Significant agricultural losses can be caused by PVX infections in several plant groups, including *Solanaceae, Chenopodiaceae, Amaranthaceae,* and *Fabaceae.* The symptoms of a potato virus infection can vary greatly depending on the specific strain of the virus, but they typically include stunting and leaf moulting. Diagnosis and treatment can be further complicated when the diseased foliage takes on an unusual pattern of yellowing. Estimates put the yield losses caused by PVX infection alone anywhere from 10% to 50%. Estimated losses in mixed infections, particularly those including PVY, range from 50% to 70%, and the yield decline is said to be more severe. To combat PVX infection, many antiviral techniques that promote plant tolerance have been developed and put into practice.

Some of these mechanisms include employing a mild virus strain to cross-protect against a more severe one, inserting genes derived from the virus, such as genes for movement proteins and coat proteins, and others, such as using the replicase gene, developing resistance through antisense RNA, and silencing genes induced by the virus itself. There is a wide range of success rates among the various resistance tactics, and these results are method dependent. There are two subtypes of resistance that these antiviral methods impart: protein-mediated and

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RNA-mediated. While the exact processes behind protein-mediated resistance remain a mystery, RNA-mediated resistance, or RNA interference (RNAi), is now widely acknowledged as a method of gene silence. In order to protect themselves from viruses and transposons, eukaryotic creatures have evolved RNA interference (RNAi), a regulatory mechanism that has persisted through evolution. Beginning with siRNA, RNA interference causes the destruction of corresponding messenger RNA.

The potato, scientifically known as *Solanum tuberosum L* ., is a vital crop in Pakistan and a promising new cash crop overall. With an average yield per hectare as low as roughly 18.6 tonnes, the current yearly production of potato tubers is about 1,105,000 tonnes. When contrasted with yields reported by countries like Australia (more than 100 tonnes per hectare) and Germany (41.6 tonnes per hectare), this yield is pitiful. The seed tuber quality is one of the most important factors influencing potato yield. While there are numerous variables that contribute to Pakistan's low potato yields, one of the most detrimental is the prevalence of viral infections such as Potato Virus Y (PVY) and the Potato Leaf Roll Virus (PLRV). Degeneration caused by these viral illnesses is seen in the potato cultivar as a decrease in vitality, yield, and overall resistance to disease. This usually happens when the potato tuber material is planted from the same source material multiple times.

Asian potato harvests have been disappointingly low, in contrast to Western Australia's typical yields of about 39.5 tonnes per hectare. It is very difficult and costly to produce enough high-quality seed potatoes that are virus-free to satisfy the growing demand. At this time, no commercially available high-yield varieties or advance lines in Pakistan have demonstrated long-term resistance to these viral infections. Reasons for this include persistent viral infections, inoculum pressure from a steady stream of diseased seeds, periodic accumulation of virus vectors, and an absence of efficient pesticides for direct field disease control. According to reports, PVY is a serious problem for potato crops in the Punjab area. It was estimated that losses in Pakistan resulting from PVY infection ranged from 40% to 70%. Incidence rates of the virus fluctuate widely from about 2% to 25%, indicating its widespread presence in the country. In its role as the prototypical member of the Potyvirus family, PVY is characterized by a lengthy, curved particle that contains positively polarized single-stranded RNA and measures

around 730 by 11 nanometres in size. Infection symptoms caused by PVY can vary greatly from cultivar to cultivar and strain to strain, ranging from a mild mosaic pattern to severe necrosis and potential plant death. Numerous plant viruses have been successfully eradicated by extending approaches that target the viruses' reproduction and movement mechanisms within host plants. Tissue culture procedures, thermotherapy, and chemotherapy are the three currently used approaches.

Although the use of heat to eradicate plant diseases has been around for more than a century, the earliest evidence of effective heat treatment for plants afflicted with viruses dates back to 1949. More than 70 viruses were found to be rendered inactive in plants with heat treatment by the late 1960s. While the specific mechanisms by which heat affects viruses remain a mystery, the conventional wisdom holds that heat treatment inhibits viral replication and movement protein synthesis by interfering with transcription activities. When meristem culture is unable to eradicate the virus from the propagative material obtained from infected plants, thermotherapy and chemotherapy are sometimes used in conjunction with one another to eradicate the virus. These methods can now potentially enhance the pace of clean plant material multiplication, meaning that disease-free plants can be made from a single plant in a short amount of time regardless of location or season. Mother plants can be treated with thermotherapy either before or after meristem extraction, depending on the situation.

There have been reports of the successful elimination of viruses, particularly potato viruses, with the use of antiviral medicines in combination with meristem culture methods. The usage of these broad-spectrum antiviral compounds has expanded beyond their original medicinal use in humans and animals. They can now be sprayed over crops or added to plant tissue culture conditions, where they are absorbed by plants and prevent viral multiplication. In addition to meristem culture methods, antiviral medicines have been tried, with encouraging outcomes in terms of virus eradication. Economically significant crops including potato, peanut, apple, and many Prunus species have been found to be resistant to viruses when treated with certain compounds like 5-Azacytidine and Ribavirin. In most cases, the rate of plantlet regeneration and the eradication of viruses are used to assess the efficacy of stem-tip culture in producing plantlets free of viruses. A high production of virus-free plantlets

depends on factors such as meristem size, culture media composition, in vitro growth circumstances, and the impacts of thermotherapy and/or chemotherapy. The generation of healthy plant material is expected to be held to a very high level when the meristem-tip culture approach is combined with a strong certification scheme. In order to address the low average potato crop yield in the country, the studies detailed below were conducted to remove potato viruses from in vitro plantlet stocks. Effective detection of PVY infections has been achieved using the serological sandwich ELISA. This method works well for estimating the number of infected potato plants quickly. In addition, the use of real-time polymerase chain reaction (RT-PCR) to directly identify PVY from total RNA taken from diseased potato leaves is also detailed in this work. One of the most effective and trustworthy methods for diagnosing PVY in field samples is an RT-PCR-based approach.

Using meristem culturing alone resulted in somewhat low plant yields (22.8% to 32.6% across all cultivars) in the context of virus-free potato cultivation. The process of producing plantlets from the meristem typically takes a few months. In most cases, stem cell cultivation is still the gold standard for curing viral infections. Because of limitations in replication and mobility, this strategy takes use of the fact that not all viruses can colonize the meristematic zone. In some cases, such as PVX and PVS, the use of thermotherapy in conjunction with meristem-tip culture was able to successfully eradicate the viruses from infected potatoes. However, this was not always the case. Heat treatments reach approximately 38 ± 2°C also at significantly lower viral concentrations. There has been some success in creating virus-free plants through the use of thermotherapy and meristem culture; for instance, *Citrus tristeza* virus and *Citrus exocortis* viroid were successfully produced. Combining the two methods has actually resulted in a 100% success rate in viral elimination, compared to a 60% success rate when meristem culture is utilized alone. Every new method for improving plant health and productivity be it through thermotherapy, chemotherapy, or tissue culture—tends to lead to an improvement in potato virus purification. Therefore, it is crucial to constantly improve protocol and methodology innovations for improved yield and quality potato production, especially in regions where viral infections are a problem. In light of food security and the sustainability of agriculture, these methods hold much potential for resolving the issues associated with potato cultivation. After

thermotherapy, we were able to achieve a maximum reduction of 46% in viral concentration in our experiment. This is due to the following reasons: One way is when the virus competes with the host cells, which divide rapidly, for the spots on the cell membrane that are responsible for making proteins and nucleic acids. A new, less favorable equilibrium between viral particle creation and destruction may emerge from this conflict. On the other hand, the viral nucleic acid's protective protein components become less stable when exposed to high temperatures. Nucleases could inactivate the virus by the appearance of transient fissures under these conditions, significantly reducing the virus concentration. Prior research has shown that temperatures between 35 and 40 ºC are effective in removing PVY from potato plants grown in a controlled environment. On the Cardinal variety, for example, heat treatment supposedly achieved a virus-free rate of 60.5% on average, with as high as 80% in rare instances. Meristem-tip culture on developing shoots combined with thermotherapy was used to successfully establish virus-free taro.

RNA Silencing and Genetic Engineering for Viral Resistance

Small interfering RNAs (siRNAs) play a crucial role in systemic RNA silencing; these RNA molecules typically include 21–25 base pairs and activate the RNA interference (RNAi) pathway. There are a number of efficient methods for starting the RNAi response, and they all involve sense/antisense RNA, shRNA, and miRNA precursors. A strong and sequence-specific response can be achieved in cultured cell lines using siRNAs, and this effect does not need the activation of the immune system. The successful induction of RNAi responses in cultured cells for target gene knockdown has been the subject of numerous methodologies. Nevertheless, there have been reports of off-target gene silencing and undesired cross-reactions, highlighting the limitations that come with siRNA targeting techniques. It was first thought that siRNAs exhibited non-specificity because of the sequence and thermodynamic features that govern their interactions. Nevertheless, these issues have been extensively addressed by employing sophisticated siRNA design algorithms, even if a large number of siRNAs are still required and should be optimised for optimal effectiveness. Here, we examined the efficacy of siRNA and shRNA in silence a local PVX isolate's coat protein gene. One of the

most potent tools in functional genomics, RNAi has emerged in the last two decades. It opens the door to unleashing cells' long-lost antiviral defence capabilities in response to targeted gene suppression. The PVX coat protein gene was selected as the target due to its role in viral accumulation, encapsidation, and systemic transmission. Research on plant resistance to PVX and related viruses has made use of viral genes, including the coat protein gene, in a number of investigations. Successful virus-resistant plant lines have been produced through the use of RNAi technology.

The most economical and effective way to create resistant transgenic plants is to identify specific siRNA fragments. Transgenic lines of tobacco, barley, and maize that are resistant to PVY, BYDV PAV, and MDMV, respectively, have all been successfully developed by the use of RNA interference. Finding the best coat protein siRNA to generate PVX-resistant transgenic potato lines was our primary goal in our previous research. Foreign nucleic acids can be delivered into eukaryotic cells through transfection, one of the few non-viral procedures that can be used to investigate relative expression levels and other preset parameters. Synthesis of target-specific siRNA, transfection of cell lines with appropriate reagents, and measurement of the percentage of gene knockdown achieved by the siRNAs constitute the standard procedure for RNAi knockdown experiments.

A phylogenetic tree was constructed by performing several alignments of the nucleotide sequence of the PVX Pakistani isolate in conjunction with other isolates. Multiple sequence alignments, phylogenetic analysis, and BLAST were used to determine the homology of the sequenced gene with previously reported sequences in the GenBank database. This showed that the highest degree of homology with the Scottish and Indian isolates was 95%. Firstly, the sequence analysis clearly shows that the PVX-CP gene sequence is quite similar to other PVX isolates. Secondly, it implies that the gene is conserved, which is useful for finding siRNA targets within the PVX genome. If the data show that the coat protein gene is the best target for a siRNA-based technique to create PVX-resistant potato varieties in Pakistan, then the hypothesis is accurate. As a source of essential carbs, dietary fibres, vitamins, proteins, minerals, and antioxidants, the potato (*Solanum tuberosum L* .) was once thought of as the fourth staple crop on Earth. It is known that potato crops can be infected by these viruses as well as two kinds of viroid. In Pakistan, the most significant viral pathogens are

PLRV, PVX, and PVY; PMTV, PVA, PVM, and PVS are also prominent. Increased vector pressure increases the occurrences of diseases caused by aphid-transmissible viruses, particularly PVY, in countries with primarily warm climates, such as Pakistan. Heat above 28 °C intensifies symptoms ranging from moderate interveinal mosaic to severe leaf mottling for PVX, a mechanically or contact-transmitted virus that is considered one of the top ten economically significant viruses globally. This is due to the fact that a combination of PVX and PVY infections in a single plant has the ability to induce more severe symptoms due to a synergistic effect.

Since the majority of viruses are kept alive through vegetative means, and better seed is not readily available in Pakistan, this becomes a significant consequence. When infected seed tubers are passed down through generations of sensitive potato cultivars, the virus quickly degenerate the seeds and causes a decrease in production. To combat these threats, scientists have created transgenic plants that are resistant to viruses by utilizing a variety of transgenic procedures. These include RNA-mediated, infected coat-protein, movement-protein, and replicase-mediated resistance. Despite these methodological advancements, only a small number of transgenic plant lines have proven to be very resistant. PTGS is now the go-to shot for transgenic plants looking to induce resistance. The process known as PTGS inhibits the expression of certain genes and viral infections in eukaryotic creatures. While the virus is in an intermediate replication step, it forms self-complementary hairpin RNA, also known as dsRNA, which induces or triggers the RNA silencing. Sections of 21–25 nucleotides of double-stranded RNA are cut into smaller pieces by endoribonucleases from the Dicer family of RNases. Next, the target RNA sequences are degraded by this siRNA and RISC combination.

RNA Silencing Strategies for Viral Resistance in Potatoes

Two more transgenic lines, on the other hand, showed CP-PVX gene mRNA expression levels lower than the control potato plants. There can be a lot of reasons why different transgenic lines have different resistance efficiencies. Prior to determining the level of gene expression, the chromosomal locus may integrate the shRNA transgene in a different way, resulting in position effects. There will be a wide range of siRNA amounts and, consequently, resistance efficiency, because host plants vary in their degrees of ploidy. Second, sense RNA

shunting and ribosome scanning are two activities that could negatively impact the correct folding of double-stranded RNA (dsRNA), which in turn could disrupt Dicer processing and the RNAi responses. Lastly, in addition to utilizing varying viral inoculation doses, it is also possible that the transgenic lines naturally express their transgene differently, impacting the level of resistance. Hence, the targeted viral gene's mRNA expression was lowered due to RNA interference against viral inoculation, made possible by inserting the shRNA transgene into the transgenic potato lines. Significant protection against PVX was achieved by seven of the nine most effective transgenic lines. These findings demonstrate the practicality of miRNA-based methods for creating transgenic potato plants that are resistant to PVX. The longest group of plant viruses, the genus Potyvirus, to which Potato Virus Y belongs, is defined by long, flexuous particles that measure around 730 × 11 nanometres. The predicted size of this virus's single-stranded RNA genome is around 9.7 kilobases, and it possesses positive polarity. Potato and other solanaceous crop infections include PVY. Tubers of potatoes suffer greatly in yield when infected with tomato and tobacco plants. Estimates put the losses in Pakistan due to PVY at 40–70%. Symptoms caused by PVY can vary greatly from a scarcely noticeable mosaic pattern to severe necrosis and early plant death, depending on the cultivar and virus strain. Because of the use of vegetative propagation in potato production, controlling PVY infection has become a challenging task in potato crops.

This approach makes the first viral infections worse, so they last longer and do more damage. Developing highly effective resistance techniques, such as the RNA silencing approach, is crucial for reducing the impact of PVY and ensuring the sustainability of potato production. As a contribution to food security and agricultural sustainability in areas where viral infections affect crops, our results lay the groundwork for future research into optimizing transgenic techniques to produce potato lines with high viral resistance. In spite of extensive research and development into potato breeding in Pakistan, no commercially viable high-yielding variety has shown any long-term resistance to viral infection. Genetic modification for resistance to the coat protein gene or the P1 gene of the Potato Virus Y can be used to circumvent such limitations, bringing up new ways for elite clones. The resistance mechanisms in virus-resistant plants are primarily protein-mediated; however, resistance given by gene silencing strategies has shown to be more effective and persistent. For transgenic plants, RNA

interference—also known as post-transcriptional gene silencing—is an essential epigenetic mechanism for viral infection resistance and gene expression regulation. This mechanism entails RNA breakdown mechanisms. Scientific investigations have proven that RNA viruses are capable of generating double-stranded RNA (dsRNA) that closely resembles the sequence of specific genes' transcribed sections. Degradation of cognate RNA molecules is facilitated by the production of small interfering RNAs (siRNAs) by means of endonucleolytic cleavage, which this double-stranded RNA (dsRNA) goes through. It has been shown that small interfering RNAs (siRNAs) are essential components of RNA silencing; typically, they consist of 21 nucleotides. There are still several limitations to using siRNA-mediated gene silencing for plant virus resistance, despite this promising future. One issue is that standards for the construction of siRNA oligonucleotides have not been finalised. This means that not all prospective siRNAs will be effective in preventing gene expression. Hence, in most cases, it will be necessary to conduct empirical testing on a panel of prospective siRNAs in order to determine which ones are the most effective.

A well-targeted screening technique is necessary before siRNA gene silencing can be fully utilized. To achieve this, we can clone each siRNA and then see how our target gene expression gets knocked down in a cell culture system. This kind of work requires a lot of human effort and takes a long time. Screening, instead, might be based on a phenotypic shift linked to gene knockdown. We have developed a transient expression-based screening strategy for siRNAs to reduce the costs associated with these laborious methodologies in mammalian cell lines. The present study aims to utilize small interfering RNAs to establish a high level of resistance to potato vein yeast (PVY) in potatoes, a crop that is economically significant yet is severely impacted by this virus. Because of this, we have devised a system to screen potential siRNAs from a large panel, with the understanding that not all of these siRNAs will prove to be very effective. Temporary expression in a mammalian cell line is the foundation of our screening process. Because of its importance in aphid-transmitted potyviruses and PVY uncoating and translation into the cell, the CP-PVY gene has been selected as a target for resistance conferring. In addition, every prior study has shown that the CP transgene segments give the transgenic plants immunity or resistance. Furthermore, transgene-derived viral dsRNA has the ability to activate RNA silencing processes that can potentially reduce viral infections. For our investigation, we

used a 480-base pair segment of the CP-PVY gene as the template to create six siRNAs. Due to a highly conserved section across the CP genes, as revealed by multiple alignments of four reported PVY strain sequences from NCBI, a portion of the CP gene was chosen over the complete CP gene template. The CP gene fragment from a local PVY strain that showed 99% sequence homology with known sequences in the GenBank database was amplified using primers developed using this conserved region. So, the discovered siRNA is anticipated to offer broad-spectrum resistance and work against multiple PVY strains, such as PVYN, PVYNTN, PVYo, and PVYC. Among these, one small interfering RNA (siRNA1) was discovered to significantly reduce CP-PVY mRNA expression by a factor of 12.25 when tested by real-time PCR in transient expression tests conducted for 24 hours in the mammalian cell line CHO-k1.

Due to the necessity for a robust line capable of providing quick expression within a limited time window, the CHO-k cell line was selected for this work rather than plant cells. This is due to the fact that cloning each siRNA in a panel before transformation and expression analysis is typically required when working with plant cells, which is a very labour-intensive and time-consuming process. In addition, our strategy utilizing siRNA is predicated on a method of "RNA silencing of target genes," which is not only present in plants and mammals but also in other kingdoms of life. The presence of small interfering RNAs (siRNAs) ranging in length from 21 to 26 nucleotides is what brings this process together, as shown in the literature. RNA silencing refers to a group of gene-silencing processes that are all characterized by the degradation of RNA at specified sequences. There are a number of known silencing events in fungus and algae, as well as post-transcriptional gene silencing or co-suppression in plants, RNA-mediated resistance to virus infection in plants, RNA interference in mammals, and maybe other phenomena. Research in genetics and biochemistry has shown that all eukaryotic species share a common mechanism of RNA silencing. Since the target gene was not expressed in CHO-k cells transfected with an empty vector, our results demonstrated that the siRNA-mediated silencing was specific to the CP-PVY gene. The theory of homology-dependent gene silencing (HDR) provides an explanation for this. Our research results were in agreement with the previously proposed processes of RNA silencing, providing further evidence that our methodology was suitable.

Genetic engineering has been used to confer resistance to viruses on potato cultivars. This is done because potatoes are a valuable crop for food production. Within the scope of this investigation, a shRNA transgene measuring 107 base pairs was expressed in transgenic potato plants in order to provide resistance against PVY. Polymerase chain reaction and Southern blotting were the methods that were utilized to be certain that the transgene was present and that it had been integrated. Because there are an excessive number of DNA pieces in the potato genome, it is difficult to trace small bits such as a 107-base pair shRNA transgene. In order to monitor the binary cassette, a special probe was utilized. The RT-PCR result demonstrated that shRNA1 was successful in providing resistance to PVY infection in transgenic potato plants. This was demonstrated by the outcomes of the experiment. This knockdown was at a level that was insufficient to give rise to either resistance or immunity in the transgenic plants, despite the fact that shRNA4 had been able to partially lower the mRNA expression of PVY in the plants. As a result, it was determined that shRNA1 was significantly more efficient than shRNA4 in terms of reducing the amount of PVY mRNA expression. It is possible that this could be explained by the fact that PVY carries the Hc-Pro RNAi suppressor, which is known to interfere with the plant's resistance response to a wide variety of viruses.

Therefore, the difference in the resistance efficacy in the transgenic potato lines may also be related to the available research, which indicates a different degree of possibly active virus-specific siRNAs. This is because the literature refers to a different degree of virus-specific siRNAs. For the purpose of identifying the targeted siRNA in the mRNA knockdown experiments, Northern blotting was performed in accordance with the approach that was described as the classical method. These analyses produced results that suggested that CP-PVY-specific siRNAs were expressed at relatively high levels in the shRNA1-containing transgenic plant, which prevented the accumulation of the CP-PVY transcript. These results were obtained by analysing the transcription of the shRNA1 gene. It was discovered that the transgenic potato plants that contained shRNA4 had the lowest amounts of CP-PVY-specific siRNA during the measurement process. This set of observations highlighted the fact that there is a reciprocal association between the knockdown of mRNA and the synthesis of siRNA. The shRNA1 construct was responsible for the greatest reduction in the expression of CP-PVY mRNA, which was found to be correlated with

the generation of more target-specific siRNA. On the other hand, the shRNA4 construct had the opposite effect. One possible factor affecting this effect is the local folding potential of the different structures. This transcript may be more sensitive to DCL1 cleavage because it can quickly fold into the secondary form, thanks to its more stable stem-loop shape compared to pro-shRNA4. Thus, it can speed up the development of RISCs and improve the efficiency of gene silencing, which is in line with earlier research. Related research has also shown that plants may effectively express dsRNAs derived from hairpin RNA or pathogen-derived long dsRNAs, both of which provide resistance to viral infections. These results demonstrate that the lengthy dsRNAs are cut into short dsRNAs, which in turn activate the RNA interference machinery and destroy the viral invader.

Achieving Viral Immunity in Potatoes through RNAi

One of the benefits of RNA silencing is the development of acquired immunity, which protects against viral infections. According to recent research, manually inoculating potato plants with PVY strains and then applying HC-Pro-specific dsRNA and siRNA resulted in PVY resistance. On the other hand, we were able to successfully create transgenic lines that had nearly cleared PVY through the protective mechanism of constitutive expression of the shRNA cassette. In addition, the newly-introduced shRNA cassette is a miRNA, which makes it more stable and provides ongoing defence against PVY. It is crucial to note that the shRNA sequence used to construct PVY-resistant plants does not originate from a virus and is not translated into a protein. The RNai pathway quickly cleaves the RNA transcript into tiny fragments, making it nearly undetectable. This work is significant because it raises the prospect of using RNAi technology to increase food security and promote sustainable agriculture by making potato plants more resistant to viruses. This technique, if well implemented, will pave the way for other transgenic crops that carry resistance to a broad array of diseases. By using RNA interference (RNAi) mediated by carefully designed shRNA constructs, a new standard for crop protection can be established, one that prioritizes efficiency and sustainability, to ensure the continued viability of staple food crops such as potatoes in the face of newly developing viruses. All things considered, our findings show that shRNA can make viruses resistant, and they also show how plant genetic engineering is changing the game for modern farming by helping us understand more about the intricate web of relationships

between plants and diseases and the molecular mechanisms that make plants immune. Protection of potato plants against PVY infection has been proven to be effective and predictable through the transgenic production of short hairpin RNA specific to coat proteins. A strong foundation for selecting the most promising potato lines with enhanced resistance to PVY can be found in the link between the level of shRNA expression and virus resistance. Reducing the hazards associated with genetic alteration, the approach avoids environmental hazards such as viral trans-encapsidation and recombination of the transgene with incoming viruses.

Transgenic Approaches for Viral and Fungal Resistance

Transgenic *Nicotiana benthamiana* developed resistance against Beet Necrotic Yellow Vein Virus, transgenic potato plants developed resistance to PVY, and numerous more investigations have documented the development of resistance in transgenic plants through RNA silencing. The adoption of siRNA-based techniques to enhance the resilience of potato crops against viral infections is supported by the efficient induction of resistance in transgenic plants. The critical need for reliable diagnostic methods and the creation of resistance to PVX and other potato crop-threatening viruses is highlighted. The successful development of a PCR-based detection approach and the detailed analysis of local PVX isolates lay the groundwork for future studies and applications aimed at enhancing potato crop resistance and production. With an emphasis on the conserved coat protein gene, our study paves the way for the use of RNA silencing techniques to create PVX-resistant potato varieties in Pakistan. This might greatly improve the long-term viability and economic viability of potato farming in the country. Our study set out to create PVX-resistant potato lines by means of RNA silencing. About 6,435 base pairs of positive, single-stranded RNA make up the PVX genome, which also includes five open reading frames. In this work, we developed a shRNA expression vector called CP-PVX that specifically targets a conserved area of the PVX CP gene. The flanking and loop sequences of the shRNA were taken from miR403, a microRNA that is extensively produced in potato plants, in order to make it more stable and useful. The efficiency of shRNA-containing constructs was evaluated by measuring mRNA expression in transgenic potato plants of the Sante variety that had been inoculated with PVX. Though it has been said that Sante, the white-skinned potato variety grown to the

highest standards in Pakistan, is resistant to PVX, cases of infection and the characteristic symptoms have been documented in the area. So yet, the causes of this infection have not been thoroughly investigated. However, it has been noted that plants' protection to certain viruses is weakened by high temperatures. Given these factors, it's possible that using a shRNA-mediated technique is the most effective way to create PVX-resistant potato plants. One of the most significant infections that reduces potato plant output is RNA virus. For example, in Pakistan, RNA viruses have the potential to cut potato output losses by 80%, whereas PVX alone is thought to cause yield losses of about 30% globally.

There has always been hope that RNA interference technology would provide a way to down regulate specific genes, whether they are viral or endogenous. One of the most common methods for developing plant resistance to viral infection is RNA silencing, which employs dsRNA at the central location. Stable expression of siRNA can be achieved in a variety of ways. Methods such as expressing siRNAs as stem-loop structures that can be processed by Dicer to produce functional siRNAs or using individual promoters to transcribe the antisense and sense strands in the siRNA duplex are among these. It has been found that expression cassettes, which generate molecules with a hairpin structure, allow for more effective gene silencing in plants compared to antisense and sense-mediated gene silencing. Our 107-bp shRNA transgenic specifically targets a conserved area within the PVX CP gene, as demonstrated in this work. As the CP gene is involved in the virus's translation, motility, and uncoating process during infection, it may offer resistance to viral infection in GM plants. We conclude that constitutive shRNA expressions are powerful enough to reduce PVX mRNA expression levels and, in the end, prevent virus infection in transgenic plants, using miR403, the most abundant microRNA in potatoes. It was possible to achieve high levels of expression of the shRNA transgene by cloning it into the Cauliflower Mosaic Virus 35S (CaMV35S) promoter-regulated binary vector pCAMBIA1301. Nine transgenic lines exhibiting consistent shRNA expression were produced from the cultivar Sante using Agrobacterium-mediated genetic transformation. The use of Agrobacterium spp. for genetic transformation enables the introduction of desired characteristics or candidate genes from other species into the genome of a host plant. Unlike plants developed through conventional breeding, transgenic plants will be resistant to

viruses and insects. We have found that after PVX inoculation, seven of the transgenic potato lines showed nearly undetectable mRNA transcription of the targeted CP-PVX gene.

Thus, it is vital to find safe and effective replacements for pesticides in crop plant disease control. Genetically engineering plants to resist certain infections through the production of suitable antipathogen components and/or biological control is another intriguing technique. Antifungal activity of chitinases against harmful fungi with chitin-containing cell walls has been the subject of extensive research. Several chitinases, such as rice class I chitinase and barley class I and class II chitinases, have been evaluated for their ability to increase plant resistance to fungal diseases using genetic transformation procedures. The effective creation of transgenic potato plants resistant to *Alteraria solani* is reported in this article. *A. solani* susceptibility was shown in transformants of the local potato variety Desiree, which were created using the barley-derived chitinase II gene. In order to prevent or reduce biosafety issues associated with chitinases produced by bacteria or fungi, barley chitinase was selected as the transgene for the experiment. Similarly, excellent anti-fungal efficacy against *A. solani* was demonstrated by a single in vitro examination. Among the many transgenic potato plants tested for transgene overexpression, the Ak-23 line demonstrated the highest levels of recombinant chitinase protein expression, a profusion of chitinase enzyme units, and strong antifungal activity against *A. solani* in both laboratory and inoculation experiments. It was inhibited by 40µg of pure recombinant chitinase protein in our experimental setting. *A. solani* had a lesser impact in inhibiting fungal growth at a concentration 10 µg lower, while it was still substantial. The fungus growth significantly decreased from 39.5% to 60.5% when chitinase was expressed in transgenic potato plants. This confirms what was previously reported in the literature, which found that larger doses of pure chitinase proteins inhibited the growth of certain phyto-pathogenic fungi. Beyond that, a transgenic potato line called Ak-23 had recombinant chitinase protein expression levels high enough to inhibit the inoculated fungus's hyphal development in a detached leaf assay.

Enhanced Disease Resistance in Transgenic Plants

The non-transgenic control plants, on the other hand, exhibited necrotic symptoms, such as the development of amber-colored to

completely black patches on the inoculation site and subsequent spread across the entire leaf surface. Because the transgenic leaves adequately expressed the recombinant chitinase protein, which hindered the hyphal expansion of the fungus, these data indicated that the transgenic leaves did in fact exhibit better resistance to *A. solani* infection. It should be noted that in previous studies using detached leaf assays on transgenic tomato plants against *A. solani* , the effectiveness of the genetic modifications in producing increased resistance was confirmed by total necrosis with chlorosis in the control groups compared to the transgenic lines.

Phytophthora infestans resistance was conveyed to the transgenic plants' leaves by additional study on the overexpression of the rice oxalate oxidase 4 gene in potatoes, as compared to non-transgenic controls. Additional evidence of enhanced antifungal action against *Sclerotinia sclerotiorum* in the transgenic leaves of canola plants has been found through transformation with the endo-chitinase gene from *Trichoderma atroviride*. The transgenic potato plants that were successfully created had a barley chitinase gene that makes them more resistant to the infection caused by *Alternaria solani.* The results show that transgenic plants can significantly improve agricultural productivity and food security without posing a biosafety risk, making them a viable alternative to chemical fungicides for fungal disease management. To minimise harm from fungal infections in crop production systems, more research in the field is considered necessary. Several methods have been used to make crop plants resistant to harmful fungus, such as expressing proteins that are involved in pathogenesis. For example, a study found that increasing the enzymatic activity of chitinase in peanuts significantly reduced the leaf spot disease. This was achieved by expressing a gene from rice. The 33-kDa wheat chitinase's antifungal action against tomato *Fusarium oxysporum* was the subject of another publication. A ribosome-inactivating protein and the barley chitinase gene conferred great resistance to the *Alternaria brassicae* pathogen, which causes *Alternaria* leaf spot, in transgenic *Brassica juncea* plants. With a reduction in hyphal development of up to 44%, this transgenic plant showed encouraging results in in vitro conditions. Under greenhouse circumstances, it delayed the onset of illness symptoms. Black leaf streak's causal agent, *Mycosphaerella fijiensis*, was the subject of an associated study that focused on anti-fungal transgenic bananas. Compared to non-transgenic plants, transgenic rice plants with the

chitinase gene inoculation showed a considerable decrease in necrotic leaf area (73-94% less) and a marked delay in disease development.

Typically, while analyzing transgenic expression, researchers utilized relative quantification to compare transgene mRNA levels to those of controls and untreated samples. Within a few time after *A. solani* inoculation, the current study found that transgenic mRNA expression was low. Over time, the expression became more prominent. Expressions that were at least 7.22 and 7.3 times more than the control group were documented. Chitinase is an anti-*A. solani* response protein, and our findings prove it. The transgenic plants had a temporary drop in transgene expression after inoculation because they were stressed. But as time goes on, the plant's mRNA expression increases, providing protection from *A. solani*. Expression of messenger RNAs was positively correlated with the amount of time that had passed from inoculation. Less fungal growth accompanied the increased expression level. The creation of transgenic sugarcane containing the chitinase III gene as a defence against *Sporisorium scitamineum* was also detailed in a different study. A 3.2-fold increase in chitinase gene expression compared to the controls was seen following an initial decrease. Another relevant study found that compared to zero hour after inoculation, the expression of the chitinase gene in *Quercus suber* roots infected with *Phytophthora cinnamomi* was three times higher at 24 hours post-inoculation. Integrating several copies of the gene is a big problem in transgene research since it frequently causes transgene silence. This is further supported by the current findings, which show that plants with a single copy insertion of T-DNA have clear advantages over those with two integrations. The expression of a transgene can be influenced by factors such as chromosomal location and copy quantity in numerous plant species. This meant that copy number variants could explain differential gene expression in many instances. We found that the transgenic plant Ak-23 had an extremely high expression level of the linked gene because it had one copy of T-DNA inserted into its genomic DNA.

On the other hand, the transgenic plant Ak-14 showed reduced expression and antifungal activity due to its two copies of T-DNA. Transcriptional activity in open chromatin areas and more compact, transcriptionally inactive chromatin regions are both possible outcomes of the random incorporation of foreign DNA during

transformation. Because of this, transgenic plants may exhibit lower and frequently fluctuating expression levels. Contrary to expectations, transgenes located in heterochromatic areas are the ones most likely to be silenced. The results show that endo-chitinase activities are strongly correlated with transgenic transcript levels. Testing the transgenic lines for fungal suppression in plants and in vitro confirmed that the antifungal effect was caused by barley chitinase protein that had been overexpressed heterologously. These encouraging results suggest that the barley chitinase gene might be used to develop fungal-resistant crop types. Cultivated potatoes are members of the genus *Solanum*, which contains around a thousand species. Only eight of the more than 200 potato species are actually grown in the wild. The potato plant, scientifically known as *Solanum tuberosum*, has been domesticated for approximately two thousand years. Its native range is the Andes Mountains in South America. Following wheat, rice and maize, potatoes have risen to the position of world's fourth most significant food crop. The potato is a staple crop, particularly in poor nations, and is eaten by almost one billion people worldwide. Developing nations' share in global potato output rose from 11% in the 1960s to 30% in the 1990s, which is an interesting trend.

Particularly RNA viruses, which infect plants and pose a significant threat to farming, fall within this category. One of the key causes for low agricultural output in Pakistan is the high number of plant-infecting viruses that have been reported there. These viruses belong to diverse taxonomic groupings. The development of sensitive and reliable detection technologies is an essential necessity in light of the prevalence of these viruses. The UK is where PVX was initially found, in *Solanum tuberosum*. Every region in the world that grows potatoes has this disease. Previous research indicated that PVX was one of the most serious viruses in Punjab, Pakistan, and that it is most commonly seen in regions where potatoes are grown. Since the early 1900s, potato farmers have understood the significance of viruses in planting material; yet, efforts to remove these viruses have only achieved limited success thus far. It is possible to determine the viral load in seed potatoes using post-harvest procedures. One such method is to grow seed tuber samples in a warmer region during winter and then use laboratory tests like ELISA to confirm the results. Nevertheless, the majority of these methods are laborious and aren't applicable to all viruses that affect dormant tubers. Furthermore, ELISA methods may

miss the presence of viruses in certain plant organs due to their extremely low concentrations.

Mechanisms and Innovations in Enhancing Crop Resistance

To summarize, we have created a system to identify highly effective small interfering RNAs (siRNAs) from a vast database of potential candidates for regulating biological processes through the targeted silencing of certain genes. This technique uses RNA interference (RNAi) to effectively silence gene expression in mammalian cell culture systems, taking advantage of their transient expression characteristics. Herein, we detail an approach that permits robust and specific results, efficient screening of powerful siRNAs from a bulk panel, and culture conditions that enable constitutive expression of target genes. In our view, small interfering RNAs (siRNAs) applied to plants to achieve sustained RNA interference will be an invaluable resource for studying gene activity in vivo and developing gene therapies for viral diseases. Agronomic crops are vulnerable to a wide range of plant diseases, which can stunt the growth of individual plants and, ultimately, reduce yields on a global scale. A decrease in crop productivity caused by these diseases might have a devastating effect on the economy, endangering both food security and sustainability. Numerous infections cause economic problems for potato farms; among these, fungal species cause the greatest damage, estimated at 40% globally. Traditional methods of fungal infection control have relied on chemical fungicides, which have been found to have negative effects on both crops and the environment. In addition to polluting the environment, chemical fungicides may have unintended consequences, such as reducing plant fertility and thereby increasing the likelihood that plants may develop resistance to certain pests.

In light of the difficulties associated with chemical control methods, it would be in a stronger position. One potential biotechnological control strategy involves inserting or transforming transgenes into plant DNA. These transgenes have fungicidal effects and could be a safer alternative to traditional treatments for fungal infections. Some of the most important fungal diseases that damage potato crops include Fusarium, a member of the Sordariomycetes class, and Alternaria, a genus of ascomycetes. A new era in disease management tactics is

about to begin, and biotechnology interventions are seen as a key component in making crops more sustainable. Chitin is an essential part of fungal cell walls and the second most prevalent biopolymer. Enzymes called chitinases break it down. Plants, animals, insects, fungus, and even viruses and bacteria are thought to contain them. Endochitinases are a type of chitinases that cleave chitin molecules into Nacetylglucosamine. They are classed as endo-β-N-acetylglucosaminidase and are part of EC 3.2.1.14. It starts with a random or single residue from the non-reducing end of the chitin molecule cleaving a 1-3 or 1-4 bond, either endo or exo. Because they target and break down the structural components of fungal cell walls, chitinases play a crucial function. Several plant species have developed resistance to phytopathogenic fungi after being inoculated with chitinases from various sources. For example, it has been demonstrated that transgenic tomato plants expressing the chitinase gene RCG3 confer resistance against *Alternaria solani* and *Fusarium oxysporum.* Liu et colleagues. recently demonstrated that tobacco plants may express the endochitinase genes CHIT33 and CHIT42, which were cloned from Trichoderma harzianum. Tobacco plants that have been genetically modified to withstand fungal infections have been discovered. Based on these data, it seems that chitinase-producing transgenes could be a good option for transgenic plants looking to build resistance to fungal diseases. The loss of crop yields is caused in part by diseases caused by phytopathogenic fungi, which are among the most common microbial pathogens. Various fungal, bacterial, and viral agents infect potatoes, which are among the world's most important food crops. Fusarium wilt, caused mostly by *Fusarium oxysporum,* and early blight, produced mainly by species of *Alternaria solani,* are the two most significant fungal diseases affecting potatoes. Modern agriculture's heavy and irresponsible use of artificial fungicides to combat a wide range of fungal diseases has raised major sustainability concerns. Regarding this, a more sustainable solution could be to genetically modify plants to withstand specific diseases.

Microbes with proven inhibitory action against infections are another potential source for disease-resistant genes. The latter are enzymes that break down cell walls; they are made by chitinase genes, which are typically activated by chitin and found primarily in mycoparasitic fungus, such as *Trichoderma* species. A variety of economically significant phytopathogenic fungi have been found to exhibit strong antifungal action against these. It has been observed that fungal

chitinases can break down the hyphal tips and the hard chitinous walls of fully developed fungal hyphae. Several studies have shown that chitinases can be useful in protecting plants. One usage of barley was to create transgenic potato plants that were resistant to the soil fungus *A. solani* by using the plant chitinase. Another strategy involved creating transgenic sugarcane plants with resistance to the Sugarcane Mosaic Virus by overexpressing the plant chitinase gene. To combat *Fusarium* wilt and early blight, we set out to create transgenic potato lines that will express the endochitinase gene. This is why two lines were produced from the potato Desiree genome that had a whole 1000-base pair chitinase gene. Multiple studies have shown that proteins involved in pathogenesis may withstand harmful fungal infections in agricultural plants. In order to make potatoes resistant to *Rhizoctonia solani* AG-3, the chitinase and β-1,3-glucanase genes, which originate from mycoparasitic fungi, were introduced into the potato plant (*Solanum tuberosum* cv. Savalan). Similarly, canola's resistance to *Sclerotinia sclerotiorum* was increased through genetic transformation with the endochitinase gene CHIT33 derived from *Trichoderma atroviride*. There were no abnormalities in the development of either of the potato lines that were engineered to include endochitinase. This confirmed what previous research had found: that endochitinase expression had no negative impact on the biological development of plants. This lent credence to previous research that found no negative effects from expressing endochitinase in broccoli plants. The current study's findings corroborate previous research showing that transgenic potato lines overexpressed the barley-derived chitinase II gene, leading to a seven-fold increase in transgene mRNA expression and sufficient resistance against *A. solani.*

In independent pot tests, the resistance to infections caused by *Fusarium oxysporum* and *A. solani* was assessed in the two transgenic lines that expressed the endochitinase. Even after 30 days of application of the infection inoculum at 3-day intervals, the transgenic plants showed a significant degree of protection against the inoculated fungal pathogens. Interestingly, following pathogen inoculation, transgenic mRNA expression was significantly increased and maintained this level for up to one month. According to these findings, the transgenic plants' increased resistance to the pathogens was due to the high amount of endochitinase expression in those plants. There is an indirect mechanism of action for chitinase in plants in addition to its chitinolytic activity. Fungal cell wall degradation releases elicitors,

which are glycosidic chains, which stimulate plant defence mechanisms. Our study's findings lend credence to the idea that endochitinases, when combined with recombinant technology, could be a powerful weapon in the fight against fungal infections in plants. On an area of 161.9 thousand hectares, Pakistan grows potatoes (*Solanum tuberosum L* .), a major cash crop that yields 3507.1 thousand tonnes each year.

There are many vitamins, minerals, carbs, proteins, and dietary fibre in this nutrient-dense tuber crop. Nevertheless, potatoes are infamously vulnerable to a number of viral infections; over 37 viruses have been documented to spontaneously infect potatoes grown in a controlled environment. Potato Virus Y, Potato Virus X, and Potato Leaf Roll Virus are among the most commonly found viruses that attack this crop. Members of the genus Potyvirus include PVY. A 9.4-kilobase genome coding for two open reading frames is constructed of single-stranded positive-sense RNA. Mechanical vectors, most notably the aphid species, are the principal vectors for the non-persistent transmission of PVY. When plants are infected with PVY, either alone or in combination, it can cut yields by 90% and have a devastating effect on plant production and tuber quality. A rapid strategy for effective management of plant diseases is urgently needed in light of this massive decline in agricultural yield and quality. One potential approach is the use of genetic engineering to increase plant resistance to viral viruses. An efficient way to enhance resistance against two significant fungal infections, *Fusarium oxysporum* and *Alternaria solani,* is to introduce chitinase genes into transgenic potato lines. Applying recombinant technology to improve plant defense systems is justified by the efficiency of developing such transgenic lines and the importance of chitinases in promoting disease resistance. Due of the widespread nature of fungal pathogens and viral illnesses affecting agricultural crops, ongoing If contemporary agriculture is to achieve its twin goals of food security and sustainability, research into resistance mechanisms and the development of genetically modified crops are absolutely necessary. Infection of a potato clone by this virus quickly becomes widespread within subsequent generations of that clone due to vegetative propagation, leading to degeneration. So, secondary infections are usually worse than primary ones when it comes to this virus. The potato's ability to propagate itself vegetatively further complicates chemical control. Thus, the most effective strategy for treating potato viral infections is host resistance. Traditional resistance

breeding in potatoes has been hindered by inherent problems in handling segregation patterns for quite some time. The pathogen-derived resistance (PDR) strategy has been extensively used to build virus resistance in transgenic plants, and it is a very promising approach that is around 2.5 years old. Although its implementation on a worldwide scale has been limited, scientists have had mixed success in creating potato virus Y (PVY)-resistant plants using several techniques, including protein-mediated ones.

The field of down-regulating a target gene, which might be virally or endogenously derived, has recently seen exciting new developments in RNA interference technology, with a particular focus on post-transcriptional gene silencing in plants. Plant transposon gene storage (PTGS) is a virus and transposon defence system that has persisted through evolution. The presence of double-stranded RNA (dsRNA) or self-complementary hairpin RNA intermediates produced by viruses during replication has been a significant inducer of RNA silencing therefore far. Enzymes called Dicers convert dsRNA into siRNA that is 21–25 nucleotides long. To facilitate the breakdown of target RNA based on its sequence, the generated siRNA subsequently binds directly to the RISC. As a protective mechanism against viral infections, plants reportedly produce siRNAs. The silencing machinery that confers resistance against viral assaults relies heavily on miRNAs as well as siRNAs. To specifically target the conserved areas of the PVY coat protein genes, two shRNA constructs were developed for this investigation. The idea was that by incorporating sequences from the highly active regulatory miR403 microRNA, which is known to be expressed in potato plants, a pro-shRNA construct could express likely effective siRNAs in a hairpin form, which would increase the construct's stability and resistance.

Both of these were expressed via the CaMV35S promoter, and then they were transformed into potato plants through the process of Agrobacterium-mediated transformation. The effectiveness of shRNA constructions was evaluated by investigating the suppression of PVY mRNA expression in transgenic potato plants that had been infected with PVY and by identifying the presence of target-specific siRNA that had been expressed, as demonstrated by northern blot analysis. It is possible that this shRNA-mediated strategy will provide a glimpse into the methodology that can be utilized to genetically create PVY-resistant potato plants. When it comes to producing virus-resistant

plants through the use of RNA silencing, the majority of the previous attempts were based on the expression of lengthy pathogen-derived dsRNAs that were approximately 300 base pairs in length. Upon entering the host cells, these double-stranded RNAs (dsRNAs) trigger the induction of the RNA interference (RNAi) machinery, which is responsible for the degradation of the viral mRNA into siRNAs. These siRNAs are considered to be hallmarks of gene silencing and provide the host plant with an active defence mechanism against viruses. There is a positive relationship between the presence and abundance of virus-specific siRNAs and virus resistance, which has been supported by a number of findings. This finding validates the fact that siRNAs are, in fact, the molecular indicators of active gene silencing. As a result, the presence of siRNAs in plants is able to detect the level of resistance that is conferred against specific viruses.

In addition, microRNAs are a subclass of tiny RNA effector molecules that are responsible for orchestrating post-transcriptional silencing. Their primary role is to reduce the amount of gene expression that is expressed in plants. As a result of the fact that siRNAs and miRNAs have the properties of site-specific cleavage of their target mRNA, inducible gene silence, and interference with viral infection, both of these types of RNAs play very significant roles in the context of plant defence. Recent research has uncovered the function that microRNAs play as components of plant resistance responses, specifically in the process of antiviral silencing. The primary purpose of this research was to provide evidence that RNA interference technology has the potential to facilitate the creation of virus resistance in potato with practical applications. It was hypothesised that a small interfering RNA (shRNA) directed against the gene that codes for the PVY capsid protein would have a significant antisense effect on the resistance of viruses.

Potato, which is one of the most important crops for food production, is severely afflicted with a number of viruses, including PVY, PVX, and PLRV. Together, these factors have a considerable impact on yield. It is anticipated that the successful generation of transgenic plants that are resistant to viruses of this kind will result in a significant improvement in agricultural yield. In the current study, two distinct shRNA vectors that were driven by the CaMV35S promoter were utilized in order to express two distinct types of artificial shRNA that were specific to the CP-PVY gene through the use of potato plants. It was previously

established that the expression of hairpin-like RNA that included transgene sequences had the capability of facilitating the suppression of transgenes in plants in a manner that was both highly efficient and specific. It has been demonstrated that small interfering RNAs (siRNAs) operate through the same mechanism as microRNAs (miRNAs) in that they interact with the messenger RNA (mRNA) of the virus that is invading the host in order to strengthen the findings of our study. MiRNAs, just like siRNAs, have been shown to be processed by Dicer enzymes and incorporated into RISC, which makes it easier to silence target genes. This has been proved through research.

Advanced Detection and Targeted Eradication of Potato Viruses

The three main potato viruses—PVX, Potato virus S, and Potato leaf roll virus—were improved detectability with the development of RT-PCR. Finding and characterizing PVX strains and genotypes in the area was the goal of this work. We accomplished this by RT-PCR amplification, cloning, and sequencing the CP gene. There is currently no documentation of the sequence that has been presented here based on DNA sequencing and homology research. It is novel. This work set out to develop a local PVX strain-specific RT-PCR-based molecular detection technique. Leaf RNA from PVX-infected potatoes was isolated and cloned into the pGEM-T vector, yielding a 613 bp RT-PCR product. Automated DNA sequencing technology was used to sequence the entire gene from start to finish, utilizing the PVX clone as a template. It was found that PVX isolates from Pakistan shared 99.7 percent similarity with previously reported PVX gene sequences in the database when the nucleotide sequence of the PVX-CP gene was compared with the PVX sequences obtained from GenBank for phylogenetic analysis. The most common method for detecting viruses in plants is ELISA, which has several advantages including speed, specificity, and robustness. One limitation of serological methods is the lack of available antisera, and another is the uncertainty surrounding antisera derived from virus combinations. In order to identify potato diseases, RT-PCR became an important tool. It is easy to use RT-PCR in separate labs once the general procedures and primer sequences are determined. Since the nucleotide sequences of numerous plant pathogens are publicly available, polymerase chain reaction (PCR) assays have been developed to identify and diagnose a number of

viroids, viruses, and other diseases. As an alternative to more conventional means of diagnosis, sensitivity has been demonstrated via several studies to be an effective tool for RT-PCR identification of viroids belonging to the apple scar skin group. The purpose of this research is to try to figure out how to use real-time polymerase chain reaction (RT-PCR) to detect PVX for commercial use. Primers derived from highly conserved sections of the PVX coat protein gene were constructed using gene sequences that were already accessible in the database. A strong level of homology with the USSR isolates was determined by successfully sequencing the PVX coat protein gene and assessing its homology with existing sequences in the GenBank database using the BLAST analysis. As part of creating healthy planting materials, it is necessary to develop diagnostic tests that can identify plant viruses. Conversely, the improvement of potato output is largely dependent on the development of efficient detection systemsWe optimised the RT-PCR conditions for PVX detection in in vitro potato leaves. It shown that the coat protein gene is an effective target for detecting viruses. Almost every country that grows potatoes has this virus, making it one of the most important ones. Seed tubers will suffer greatly in quality if phloem necrosis develops. Because of this, it is an essential quality for healthy seeds. Infected plants typically provide much lower yields than uninfected ones due to the reduced formation of small-sized tubers.

Due to its extensive distribution and generally reported incidence rates ranging from fifteen percent to sixty-five percent, PLRV is thought of as one of the most significant viruses affecting potato crops in Pakistan. As an explanation for the quick accumulation in Punjab's spring potato crop, an infestation of the aphid vector *Myzus persicae* was cited. Recent studies have shown that PLRV is one of the most recent and commercially important viruses impacting Pakistan's potato crops. PLRV is a member of the *Luteoviridae* family and is thought of as the prototypical Polerovirus. About 6 kilobases long, the virus's genome is mono-partite and non-polyadenylated RNA. Several features of PLRV have been uncovered by investigations of its fundamental features, which were carried out on a field isolate from Pakistan. Tissue culture virus removal from seed potatoes, including PLRV, necessitates a precise and accurate detection technique. The low viral content in plant tissues and its limited immunogenicity were the primary reasons why the majority of the attempted serological studies failed to reliably identify PLRV. It has been extremely challenging to detect PLRV

infections in previously infected tubers, whether they are in their dormant or post-dormant stages. For the purpose of ELISA detection of PLRV in dormant potato tubers, dormancy breakdown is an essential prerequisite. RT-PCR is a new method that could be more sensitive than traditional methods for detecting viruses in inactive tubers, including PLRV. The purpose of this research was to establish a particular RT-PCR methodology for the identification of PLRV in Pakistani cultivars' latent tubers.

Optimizing RT-PCR for Sensitive Detection of Potato Viruses

A molecular detection tool based on real-time polymerase chain reaction (RT-PCR) can accurately detect PLRV infection on both dormant tubers and foliage with relatively small sample volumes. Actually, compared to other tools, PCR is superior because of its very high sensitivity and the fact that it rarely uses radioactive probes. Prior research demonstrated that apple scar skin group viroids may be successfully detected by RT-PCR amplification of total nucleic acid extracts from diseased pome fruit trees. With this technique, amplification of any genomic fragment is possible, given the right primers.

We have already presented the results of a successful molecular detection of PLRV from tubers that have been quiescent for four months and preserved at 20-25°C. Additional results demonstrated that compared to other potato viruses, the PLRV titer in potato leaves and tubers is significantly lower, as confirmed by the two indicator plants and ELISA. In the instance of Potato virus Y, for instance, RT-PCR detection might be achieved with nucleic acid templates made from tuber extracts diluted to a 1:4000 ratio. Nevertheless, contaminated cultivar tuber extracts did not show any detectable PLRV beyond dilution. It has been observed that the titer of PLRV is lower in tubers compared to leaves, which is consistent with studies in plants infected with PVY.

A number of molecular approaches have been devised for the purpose of detecting and controlling viroids and viruses affecting potatoes. A common belief is that PCR is more sensitive when the amplicon is smaller. The specificity and sensitivity of the test were enhanced by

limiting the size of the PCR product to 400 bp in this investigation. One method that has been suggested as a good fit for detecting PLRV in early tubers is Real-Time PCR. The method outlined here is more practical and less expensive than Real-Time PCR, which requires expensive chemicals and equipment. A dependable molecular detection technique based on real-time polymerase chain reaction (RT-PCR) was developed and refined for use with locally grown potato varieties in this investigation. In order to improve seed potato quality and, in the long run, increase potato crop productivity, this created technology can be utilized for preliminary viral load diagnosis in potato tubers. In terms of global food production, potatoes (*Solanum tuberosum L* .) rank fourth, behind only rice, wheat, and maize. Worldwide, 53,666,000 hectares of potato land are used to produce 374.4 million metric tonnes of potatoes each year, according to the FAO. Pakistan produced 3,491.8 metric tonnes of potatoes from an area of 159.3 hectares. Potato Virus X is widely considered to be a very dangerous viral infection that can damage potato crops all over the world. When it comes to its genetic composition, PVX contains a single positive-strand filamentous body RNA genome that is 515 nm long. Five genes make up PVX's whole genome: one for the 3′-proximal coat protein, two for proteins involved in viral movement, and one for the 165 kDa replicase protein, which is produced by a 5′-proximal gene. Infected potato plants display symptoms such as restricted development, reduced size of leaflets, mild leaf mottling, and severe mosaic patterns.

Plants belonging to the *Solanaceae* family serve as its hosts. In the UK, yield losses for PVX and PVY, along with PLRV, are estimated to be between £30 million and £50 million in an "average" year for infections. Typically, during a PVX infection in Pakistan, the potato harvest loses about 5-10% of its yield. So yet, there is no evidence of any new studies reporting revised figures on yield losses due to PVX in the reviewed literature. Its fast genome mutation poses a serious problem for conventional viral control methods. This is why it's important to perform in-depth analyses of the virus's genome to find its most conserved regions; thereafter, methods like small interfering RNA can be used to target these regions. One technique that has shown some potential in viral control tactics is siRNA, which is finding more and more uses in the battle against viral infections that impact both plants and animals. Therefore, cutting-edge approaches to monitoring and managing PLRV and PVX are highly sought for. When it

comes to detecting PLRV in dormant potato tubers, the current RT-PCR procedure is second to none, and it helps with better management methods in potato production. In order to create successful countermeasures against the PVX virus in potatoes, additional research into its genetic characterization is anticipated. The importance of this work lies in the fact that it brings to light the necessity for sufficient molecular detection methods and in the fact that novel biotechnological methodologies hold promise for improving potato crop yields. A commercially viable polymerase chain reaction (PCR) detection method for PVX was the focus of the current investigation. Molecular hybridization techniques like enzyme-linked immune-sorbent assay (ELISA) are inferior to polymerase chain reaction (PCR) because of the latter's enhanced sensitivity. PCR allows for faster diagnosis and requires a significantly smaller sample amount than other procedures. Multiple PCR detection techniques for different viroids, bacteria, and diseases are now in development, thanks to the growing quantity of plant pathogen genomic sequences. Some of the plant viruses that have been successfully detected using polymerase chain reaction (PCR) include grapevine aphid (GVA), apple scar skin virus (ASSV), and potato virus A (PVA) in unresponsive tubers. The development of a single response that could identify five different potato viruses—PVX, PVS, PLRV, PSTVd, and PVY—was also made possible by multiplex reverse transcription-PCR.

Despite claims that real-time PCR is more efficient than traditional PCR, the high expense of the necessary equipment and reagents frequently prevents its use in PVX detection. Consequently, we used the standard approach of Polymerase Chain Reaction for detection. In order to routinely diagnose potato viruses, a conventional PCR-based assay has been suggested. This method has higher sensitivity and produces findings in less time than other options. Accordingly, we employed this molecular approach for PVX characterization investigations in potato crop research. The results lends credence to the earlier findings of those investigations. The CP gene sequence of the Pakistani isolate showed a significant level of genetic similarity with isolates from all around Eurasia, including much of Europe and India. Phylogenetic study also showed it clustering closer to these than the Australian one. The PVX whole coat protein gene was sequenced using a 710 kb fragment.

Efficacy of Ribavirin, Azacytidine, Thermotherapy, and Meristem Culture

When it comes to treating viral illnesses in potato plants, thermotherapy and chemotherapy have been considered as complementary approaches. Ribavirin and Azacytidine were the antiviral medications that were utilized in our trials. Although Azacytidine reduced PVY concentration by 38.2%, ribavirin was much more efficient, eliminating 46.9% of the virus. As a result, Ribavirin has a substantially better anti-viral effect than Azacytidine. It is worth noting that the most effective concentration for completely eliminating the virus was 50 mg/L during this process. Nevertheless, it was shown that the survival rate of the treated plantlets decreased along with this rise in efficacy. There is strong evidence in the literature that ribavirin is an effective antiviral medication when used to eradicate the other potato viruses being discussed here (PVX, PVS, and PVY). Significant suppression of PVY titer has also been observed with the use of ribavirin and azacitidine. The literature states that its effectiveness in eliminating plant viruses depends on the concentration employed, the type of infected tissue, and the host plant. The antiviral drug in question has a broad range of potential uses in combating RNA and DNA viruses that infect not only plants but also animals and people. A number of potato cultivars have been able to have PVX, PVY, PLRV, and PVM eradicated with the use of antiviral medicines in in vitro cultures.

Additionally, it has been observed that a combination of chemotherapy and thermotherapy significantly improves the eradication of PVY. When ribavirin and thermotherapy were administered simultaneously, the maximum rate of PVY clearance was 64.3%; when azacitidine and thermotherapy were administered simultaneously, the viral concentration was reduced by 58.0%. Several studies have shown that by combining chemotherapy with thermotherapy, PVY can be eliminated by as much as 83%.

In the end, the sensitivity of the virus detection methods used may determine whether our data show a lower or higher PVY eradication rate compared to the literature. Other studies primarily used ELISA for virus detection, although we used RT-PCR in ours. The virus eradication indices show that the best way to get rid of the potato virus is to use thermotherapy along with antiviral medicines in the growing medium. In addition, prior research has demonstrated that thermotherapy

reduces PVY, PVX, and PVS titers when ribavirin is present in the medium. With an improvement in meristem culture achieved by combining thermotherapy and chemotherapy, our investigations revealed that the highest values for PVY elimination were 90% for potato cultivar Desiree, 93% for Cardinal, 93.4% for Diamant, and 96% for Sante. Based on what has been said and shown, it seems that a combination of meristem culture, chemotherapy, and thermotherapy can effectively eradicate PVY from potatoes. As a whole, the goals of sustainable, high-quality potato production and the prospect of obtaining virus-free seed potatoes are both advanced by the combined strategy. The efficacy of these approaches in eliminating plant viral infections highlights their significance for agricultural practices, particularly in areas that are severely impacted. Food security and agricultural output are both improved as a result

Application of RNAi and siRNA in Crop Protection and Yield Enhancement

In order to ensure that the transgene was expressed efficiently in the HepG2 mammalian cell line, we optimised the transfection conditions. To obtain high transfection effectiveness with low cellular damage in cultivated mammalian cells, the ideal environment must be identified. In order to avoid the transfection reagent-mediated toxicity from hiding the phenotypic expression caused by the target gene under study, it is crucial to maintain low levels of cell toxicity in the cell culture system. We tested a range of siRNA dosages to find the one that would provide the best knockdown efficiency without causing any unwanted cytotoxic effects. By rapidly inhibiting its expression at the post-transcriptional gene silencing stage, the CP-PVX gene was down-regulated by the application of small interfering RNAs (siRNA). Therefore, in order to generate a strong RNAi response against the CP-PVX gene, it is necessary to screen for suitable and efficient RNAi target areas. Understanding the mechanisms that regulate the efficacy of gene silencing by particular siRNAs is a gap in the current state of study. A predictor of the gene-silencing efficiency is the local structural properties of the mRNA in the targeted region, and it has been known for a while that not all siRNAs directed against cognate mRNA have similar effectiveness. Our study's overarching goal was to learn what factors affect the effectiveness of RNA interference (RNAi) and how siRNA and shRNA, which target the PVX coat protein gene, compare in

terms of efficiency. We use RNA interference to learn more about how plants and viruses interact by improving the design and delivery of small interfering RNAs (siRNAs) in order to manage viral diseases in economically important crops. Only siRNA3, which was produced from the 551-basepair location of the CP gene, outperformed the other two tested siRNAs in terms of its capacity to down-regulate the target mRNA transcripts. According to this finding, the silencing efficacy of the siRNAs evaluated in this work varies significantly. One possible explanation is that the efficiency of the RNA interference varies depending on which siRNA target mRNA is most accessible. In comparison to cells treated with siRNA2, those treated with siRNA3 exhibited the greatest amounts of siRNA synthesis.

In comparison, samples treated with siRNA1 exhibited the lowest expression of the targeted siRNA, suggesting that it may have been partially destroyed or had no effect on the target mRNA. Surprisingly, at a dose of 100 nM, siRNA2 only managed a maximum knockdown rate of 73.14%, whereas siRNA3 achieved a remarkable knockdown of 91.91% at a much lower concentration of 50 nM. Significantly, the knockdown trend remained unchanged over the first 24 and 72 hours of incubation, indicating that siRNA3 effectively reduced CP-PVX mRNA expression. Consistent with earlier research, the increased expression of target-specific siRNA following siRNA3 treatment lends credence to the idea that siRNA sequence is a key factor determining the efficacy and specificity of viral gene silencing. As far as the world's food and nutrition situation is concerned, approximately one billion people are undernourished. Developing nations, where potatoes and other staple crops are abundant, are home to many of these people. The consumption of potatoes also saw a spectacular 22 kg/capita/year growth rate between 1960 and 2005, nearly doubling during that time. Concerningly, farmers tend to cultivate potatoes mostly through vegetative propagation, which adds to the growing dependency on potatoes as a food source. The inherent risk of disease transfer from one generation to the next is a major concern with this approach, which in turn reduces agricultural productivity. Potato tubers are susceptible to a wide variety of illnesses, including those of a fungal, bacterial, or viral nature, despite their economic importance as a crop globally. In potato production systems, these harmful organisms can lead to significant yield losses.

Fungal diseases are the most common and devastating. For example, fungi like Fusarium solani and *Fusarium oxysporum* cause damping-off and wilting in potato, cotton, maize, and rice crops. Another important pathogen, *Alternaria alternata*, causes leaf spot in cereal and food crops, which severely reduces crop yields. Early blight, caused by the fungus *Alteraria solani,* is one of the most destructive pests that can infect tomato and potato crops. Early blight induced by this disease is very harmful under high-humidity environments, especially with frequent rains. Complete defoliation of host plants caused by this necrotrophic disease can result in yield reductions of up to 79%. The chitin fibres and glucan interconnected by proteins make up the bulk of the fungal cell wall, making it an inherently complex organelle. Chitin is made up of N-acetylglucosamine and is thought to be the second most prevalent polysaccharide in nature. Inflicting "morphologicalFuoring" on the fungal cell wall is a surefire way to cause cell death by putting the cell development pattern into an osmotically unstable disorder. There is an enzyme called chitinase in this chitin that helps break the β-1,4-glycosidic link hydrolytically. Chitinase, an enzyme primarily involved in depolymerisation of chitin, protects cells from fungal infections and supplies them with carbon and nitrogen. In addition to their effects on growth and development, chitinases in plants may have a significant part in the plant's overall defence systems against a wide variety of biotic and abiotic stimuli. Various methods have been employed to safeguard agricultural plants from various infections, including insects, viruses, and fungi.

To that end, modern agriculture makes use of a broad variety of pesticides, herbicides, and fungicides in an effort to drastically cut down on crop losses. Rotating crops, practicing good sanitation, applying chemical fungicides, pulling sick plants out of the field, and ensuring a steady supply of nutrients are some of the standard methods used to combat the effects of *Alternaria solani.* Despite the effectiveness of chemical fungicides, there are significant worries about the environmental impact and the development of resistance in pathogen populations due to the dose frequency of 15 to 20 applications per crop growing season, which is necessary for adequate control of early blight. One molecular strategy that has emerged in response to the drawbacks of chemical fungicides is the manipulation of proteins associated with pathogenesis to increase plant resistance to fungal infections. This is why the current study has concentrated on investigating whether the barley-derived chitinase gene can combat

the early blight-causing *Alternaria solani.* Here, the efficacy of the purified recombinant chitinase protein against *A. solani* in vitro tests determined the protein's potential. In addition, transgenic potato plants encoding the chitinase gene were engineered to inhibit the hyphal development of *A. solani* and investigate the in planta action of the chitinase enzyme. There are serious concerns about bio-safety arising from modern agriculture's reliance on chemical fungicides to protect crop plants from various fungal pathogens. These concerns include harm to consumers' health, problems with the environment, and the rise in pathogen populations resistant to fungicides.

Further Readings

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Chapter 4: Sugarcane

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Economic Importance and Challenges in Sugarcane Cultivation

Chemicals, paints, plastics, synthetic materials, pharmaceuticals, fibres, detergents, pesticides, and a host of industrial goods such furfural, dextran, alcohol, paper, chipboard, confections, drinks, chemicals, paints, and sugar are among its most important uses. India, China, and Pakistan are the world's leading sugarcane producers, accounting for almost half of the total. A variety of sugar and byproducts made from sugarcane are grown in Pakistan, where the crop has a prominent position. Also, the paper and board sector has looked at it as a potential raw material source. According to Pakistan's economic assessment 2014–2015, the country's sugar exports brought in US$171.78 million, or 0.6% of GDP, from July 2014 to March 2015. Between 2014 and 2015, around 1,141 thousand hectares were used for sugarcane agriculture. If we want to meet the rising demand for sugarcane, for instance, we need to do all the necessary things to increase the yield per acre. When compared to the global average of almost 60 tonnes per hectare, Pakistan's average sugarcane production of 46 tonnes per hectare remains significantly low. It ranges from 65 to 70 tonnes per hectare in India, a neighboring country of this nation. The low average sugarcane yield in Pakistan is thought to be caused by a myriad of factors. These include wrong cultural practices, climate variability, drought, soil salinity, weeds, planting uncertified seeds, harvesting late, applying imbalanced nutrients, rationing incorrectly, and the prevalence of fungal diseases and insect pests. Gaining optimal yield is hindered, in large part, by weeds. It squabbles with the primary crop for water, nutrients, and sunshine, all of which are essential for growth. Most experts agree that weeds pose the greatest threat to

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crop establishment within the first 90 to 120 days after planting due to their ability to compete for fertilizer nutrients. After this pivotal point, sugarcane obtains an advantage over weeds by suffocating them.

Pesticides pose a danger to human health and the environment on a global scale. When looking at global agricultural productivity, pests and diseases are estimated to be responsible for around 37% of yield loss, with insects accounting for roughly 13% of that loss. Pakistan has recorded 61 insect species, out of a global total of 1300 that are known to infest sugarcane crops. The two most frequent species of stem borers in Pakistan, *Chilo infuscatellus* and *Chilo auricilius,* are responsible for losses ranging from fifteen percent to thirty-six percent. Damage estimates range from 10% to 15% due to the most significant borers, *Scirpophaga excerptalis* and *Scirpophaga novella*. Root borer *Emmalocera depressella* can infest crops to a level of 10–20%. While the Gurdaspur borer (*Acigona steniella*) is responsible for around 20% of crop damage, the sugarcane leafhopper (*Pyrilla perpusilla*) can cause as much as 25%. The devastating *Aleurolobus barodensis* whitefly can infest up to 80% of leaves and reduce crop yields by 15% to 25%. Mealybugs (*Saccharicoccus sacchari*) and black bugs (*Cavelerious excavatus*) are two other minor pests that attack sugarcane all year round. There is a yearly uptick in the application of herbicides and insecticides due to the increased demand on growers to prevent crop losses caused by these pests. Transgenic plants are protected from significant *Lepidopteran*, Dipteran, and Coleopteran pests by crystalline proteins produced by using the cry genes from *Bacillus thuringiensis*. A number of other Cry gene variants, such as Cry1Ab, Cry1Ac, Cry1Ac + Cry1F, Cry2A, and Cry1Ac+ Cry2A, are now present in Bt crops and have shown promising results in controlling *Lepidopteran* insects. It was found that transgenic crops require five to twelve pesticide applications fewer than non-transgenic crops during the season. From a chemical perspective, glyphosate is also known as N-phosphonomethyl glycine. It is an effective herbicide for controlling weeds since it is broad-spectrum and very active. An essential component of plants' shikimic acid system, this herbicide blocks the EPSPS enzyme, which in turn contributes to the production of essential aromatic amino acids.

Tolerance to glyphosate can be maintained in transgenic plants by using genes that encode a soil bacterium form of the EPSPS enzyme. A number of factors, including glyphosate's non-selective method of

action, minimal toxicity to humans and other non-target organisms, and its distinctive way of action, have led to its widespread use. Reaching "better self-sufficiency" in sugar production by managing cane borers and weeds and "meeting the demands of an ever-growing world population" were the overarching objectives of this initiative. Despite a number of obstacles, including cane borer infestations, weed competition, drought stress, and virus infections, the end goal of sugarcane cultivation is still high yield. In order to reduce cane borers and weeds, the present study used genetic transformation to develop insect-resistant codon optimised genes (CEMB-Cry1Ac + CEMB-Cry2A) and a glyphosate-tolerant gene (CEMB-GTGene) in sugarcane. Pakistan has ideal growing conditions for sugarcane; however the country ranks fifteenth in sugar output while having the fifth-largest producing area. This indicates that the yield per unit area is low. The monocot species places a premium on the development of a trustworthy and repeatable transformation technique. The overarching goal of this research is to develop a method that will allow transgenic plants to regenerate sugarcane calli while simultaneously protecting them from cane borers and the herbicide glyphosate. Tissue culture tests were conducted using four local elite varieties: CPF-246, HSF-240, CPF-213, and CPF-234. Callus induction makes use of these immature leaves as explants because, according to multiple studies, they are great places to get the embryogenic callus that is crucial for sugarcane genetic engineering. In this investigation, embryogenic calli were generated from each of the four types using a callus formation media that included 2,4-D. Casein, which increases the sugarcane calli's embryogenic competency, was added to the medium to further enrich it.

Genetic Engineering for Disease and Virus Resistance

One of the most important plant crops, sugarcane is typically produced for its sugar-containing stems and propagated vegetatively. Sugarcane is the source of white sugar for around 75% of the world's supply; the stems of mature sugarcane plants may store 12–16% of their fresh weight and around 50% of their dry weight as sucrose. One of the many challenges faced by sugarcane farmers is mosaic, a devastating and significant pest that causes significant economic losses in sugarcane fields as well as those of maize, sorghum, and other graminaceous plants. Sugarcane yield losses in Pakistan due to mosaic disease have been estimated to be 10% to 32%, with an impact of 6%

to 10% on sugar yields. Sugarcane mosaic virus is a well-known pathogen that reduces plant photosynthetic capacity, which in turn reduces sugar content and production. The scenario of this viral infection is complicated by the presentation of symptoms and yield loss caused by the presence of various strains. Within the family Potyviridae, the genus Potyvirus includes the sugarcane mosaic virus

Ten fully formed proteins, numbered from N-to C-terminus, are encoded by the virus's single-stranded RNA genome. Coat protein (CP), viral protein genome-linked (VPg), first 6K protein (6K1), cylindrical inclusion protein (CI), second 6K protein (6K2), helper component proteinase (HC-pro), and P1 are the names of these proteins. The improved virus transport across cells is also associated with a different protein, P3 N-PIPO, which is engaged in a translational frameshift within the P3 cistron. The sugarcane mosaic virus has a high mutation propensity because of its huge population size, short generation period, and RNA-dependent RNA polymerase's low proofreading ability. This led to the development of various viral strains, including A, B, D, and E. Aphids are the primary vector for the sugarcane mosaic virus, and the coat protein is an essential component of this vector. This coat protein-encoding gene is essential for the virus's local and systemic mobility, genome coating, and replication; it is highly multifunctional. In addition, the coat protein gene sequence is frequently used to differentiate between SCMV strains. Sequence-specific post-transcriptional gene silencing by RNAi interference has long been thought of as a conserved biological process in both plants and mammals. The process begins with double-stranded RNA, a molecule that is sequence-matching to the gene that has to be silenced. The process of RNA interference (RNAi) begins with dsRNA or hpRNA, which are then converted into siRNA duplexes, which are usually 21-24 nucleotides long, by proteins called Dicer or DCL. Ribonuclease III cleaves longer double-stranded RNAs into smaller interfering RNAs, which are 21–22 nucleotides in length. These RNAs mediate the degradation of messenger RNA based on its sequence. The RNA-silencing mechanism has dual functions in plants: first, as an inherent defence mechanism against viral pathogens; second, as a regulator of the expression of endogenous genes involved in different stages of development. Researchers used RNA interference to silence the sugarcane mosaic virus's coat protein gene in transgenic sugarcane varieties.

RNA silencing plays a crucial role in plant defense mechanisms by regulating the expression of endogenous genes. The use of short interfering RNAs, typically ranging in length from 21 to 26 nucleotides, is an evolutionary conserved natural defense mechanism. Its description led to the creation of virus-resistant transgenic plants that express microRNAs, which are manmade RNAs that target certain viruses. In one of our early studies, we use RNA interference to silence the expression of the sugarcane mosaic virus in farmed types. Because this gene is involved in important steps following virus un-coating, translation, and transmission by aphids, cDNA encoding the coat protein of sugarcane mosaic virus was used. According to supporting evidence, plants that have had a segment of their coat protein transgene introduced may develop immunity or resistance. Another option is the use of dsRNAs produced by viruses, which can effectively inhibit viral infection by RNA silencing. For the purpose of designing siRNAs and, by extension, shRNAs, a 456-bp segment of the coat protein was selected as the template. A highly conserved area could be detected by matching four sequences of sugarcane mosaic virus, which is why a fragment of the coat protein was chosen instead of the full-length one. The primers that were designed for RT-PCR amplification from a local sugarcane mosaic virus strain share 96% sequence similarity with known sequences in GenBank; this conserved area was utilized for this purpose. Consequently, it is anticipated that the resistance offered by any specific siRNA or shRNA will be effective against a variety of sugarcane mosaic virus strains, including A, B, D, and E. We chose four specific small interfering RNAs (siRNAs) targeting the coat protein to achieve a greater level of inhibition against sugarcane mosaic virus. Although there has been some success with antisense and sense-mediated gene silencing strategies in developing virus resistance, it appears that hairpin-like compounds are more efficient at promoting silencing. After being transported to the plant cells, the RNAi cassette is produced, and the resulting double-stranded RNA molecule degrades particular mRNA sequences. Plasmid administration encoding tiny interfering RNA (shRNA) and subsequent processing is one generic approach to the long-term production of RNA interference. With this method, the drawbacks of siRNAs, which include their inability to suppress specific genes in cells or entire organisms over the long term, are no longer an issue. As negative regulators of plant gene expression, microRNAs are another type of RNA silencing effectors. As a defence mechanism against pathogen infection, plants employ small RNA silencing. Thus, the premise of this

research is that sugarcane can be further protected from the sugarcane mosaic virus by expressing shRNA as a highly expressed microRNA. Unlike the CaMV35S promoter, which has been demonstrated to result in relatively low expression levels in monocot plants, the polyubiquitin promoter was used to drive the expression of the shRNA fragments. Methods for plant transformation are among the most powerful ways to introduce desirable features from other species by knocking out or overexpressing genes. Obtaining the attributes associated with these types of features could be challenging or impossible using conventional breeding methods. Virus resistance, pesticide resistance, or insect resistance is just a few examples.

The transgene is inserted into the plant genome through illegitimate recombination; nevertheless, different transformants will have different copy numbers, chromosomal locations, and arrangements of tandem repeats, including the correct orientation. As a result, gene expression can change depending on factors such as the gene's nucleotide sequence, the promoter type, the location of the transgene's insertion in the DNA, the number of copies of the transgene, the transient cellular environment, and many external environmental factors. Sugarcane undergoes stable and efficient transformation when embryogenic calli are bombarded. Sugarcane transgenic lines exhibited varying degrees of mosaic virus resistance. The reactions of shRNA2 and shRNA4 to different cultivars are distinct, and the levels of resistance seen are also not constant. The presence of certain virus-encoded silencing suppressors, such as HC-Pro, a potyvirus-specific RNA interference suppressor, is likely to blame. Variability in plant resistance could be attributed, in part, to microRNA stability, selectivity to target mRNA, and expression levels. Results from developing transgenic tobacco lines to provide resistance against the cucumber mosaic virus were comparable to the ones shown above; however the results were not uniform. Finally, our study's findings showed that RNAi can be initiated after virus inoculation using short RNAs expressed by transgenic sugarcane plants. Here, pre-shRNA pieces that have been incorporated are processed to produce the small RNAs. One of the two pre-shRNA constructs utilized in this investigation demonstrated higher resistance in one of the sugarcane cultivars that were examined. The results show that the transgenic sugarcane plants were effectively protected from SCMV infection by either shRNA2 or shRNA4. According to these results, the microRNA technique has the potential to be a useful tool for creating transgenic

sugarcane plants that are immune to SCMV infection. Manipulating *Saccharum officinarum L*.'s metabolic pathways is one of the most promising ways to produce valuable chemicals from this crop. The synthesis and processing of biomolecules of use have been made possible through various genetic engineering techniques. Scientists are taking an interest in sugarcane products for a number of reasons, one of which being the increasing demand and value of these products in various food businesses. There is also an upward tendency in their more common uses as feedstock, bioenergy feedstock, and raw feedstock in numerous food processing units. Evidently derived from sugarcane juice and used as a main ingredient in many foods, sucrose (SUC) is a highly prevalent non-reducing disaccharide. In plant vascular bundles, sucrose also serves as a transport medium for carbohydrates.

Optimizing Sugarcane Sucrose and Sugar Isomerization

The sucrose molecule typically contains a variety of isomers due to its status as a glycosidic product generated by connecting GLU and FRU. Although they differ in structure, all of these isomers have the same functional purpose. Among these, TH and IM stand out due to their widespread use in contemporary food production and their recent surge in popularity as natural sweeteners. Honey, jam, and jelly are examples of nutritious foods that include these isomers. It is an inherent property of certain microbial groups to undergo sucrose isomerization through microbial fermentation. The microbes have an advantage over their competition in the microbial kingdom due to this distinctive isomeric property. Microbes use a variety of enzymes to convert sucrose into more valuable forms, such as isomaltulose synthase (IM) and trehalulose synthase (TH). One such enzyme is sucrose isomerase (SI). A lot of work has gone into finding ways to employ genetically engineered proteins as expression vectors in biochemistry so that we can make items that people actually want. Isomers of sucrose differ from the parent molecule in several ways; for example, they are less acariogenic and more acid-stable, and they have a lower glycaemic index. In particular, IM and TH are well-suited to diabetic patients because to their slow digestion, which allows them to avoid dangerous spikes in blood sugar levels. Trehalulose outperforms conventional sucrose in terms of solubility, yet all sucrose isomers offer many useful characteristics.

Although previous research has also shown that SI may effectively convert highly soluble sucrose to trehalulose, increasing consumer value, the enzymes used for this process are often quite expensive and are derived from microbes. This means they aren't as versatile. In addition to all the benefits listed above, SI enzymes work in low-free-energy, multi-step pathways and do not require a cofactor to perform at their best. The sucrose isomerase enzymes that have been discovered so far all share a common TIM-barrel family with thirteen components that originate from glycosyltransferases. These SI enzymes exhibit a wide range of variation in terms of their modes of action, energy requirements, conversion kinetic rates, product ratios, and, under ideal conditions, the amounts of isomaltulose and trehalulose that can be produced. Reportedly, the microbial strain with the highest levels of output is *Pentoea dispersa* isolate UQ68J, which has a conversion rate of 91%. In contrast to isomaltulose produced from sucrose, *Pseudomonas mesoacidophilia* has shown to be more efficient in producing greater trehalulose ratios. Additionally, whitefly species like *Bemisia argentifolii* may be producing it, according to unconfirmed research. Databases now have sequences of numerous microbial sucrose isomerase genes.

New possibilities for acquiring the SIGs from their natural sources were made available by codon optimization and gene characterization. This allowed for chemical production of the genes before they were cloned into the recipient plant genotypes. A recent article highlighted the use of target SIGs within combination dual promoters in the pCAMBIA1301 vector as a novel and promising approach to maximize the conversion of sucrose into isomaltulose and trehalulose. These isomers are more efficiently supplied from the cytoplasm to the developing tissues of juvenile plants because they metabolize more slowly in plants. Trehalulose has received less attention from scientists in the past compared to isomaltulose, which has been the subject of much success in sugar generation through effective isomerization. The food biotechnologist has failed to adequately identify and develop this sugar isomer, and it has been neglected throughout the years. Trehalulose can be synthesised in culms rather regularly by a variety of bacterial strains that possess the biochemical tools necessary to produce the sucrose isomerase enzymes. This research looks at how sugarcane's cytosolic and vacuolar compartments express a modified version of the trehalulose synthase gene II (ThSyGII).

Researchers tested the efficacy of using polyubiquitin pUbi and Cestrum yellow mosaic virus CmYMV single and dual promoters in a combinatorial expression study. In both cases of transgenic lines, mRNA expression measurement was performed using real-time polymerase chain reaction (RT-PCR) on stem and leaf tissue. Sugar recovery percentages were calculated in comparison to control non-transgenic lines using Brix readings and HPLC-based quantification of sugar contents. Since higher SRP is directly related to greater profitability for growers.

Herbicide Resistance Advancements in Sugarcane

During the 2006–2007 growing season, weed competition was expected to cause a 9.84–54.98 percent drop in sugarcane yield. As they mature, weeds release a number of harmful compounds that stunt the plant's progress. In Australia, weeds are expected to cost around $70 million per year in management costs and productivity losses. Sugarcane faces competition from four main weed classes. Some examples of these plants are vines, broadleaf, sedges, and grasses. Glyphosate, more well known as "Roundup" and other Organophosphorous herbicides, is the principal tool used to manage weeds in sugarcane fields nowadays. The creation of herbicide-resistant crops is one of the most significant scientific achievements in the fight against weeds. As a result, scientists are currently trying to find ways to introduce herbicide-resistant genes into crop species so that they can withstand a wider range of herbicides. To make plants more resistant to herbicides, scientists have turned to genetic engineering. Sugarcane yields can be significantly increased by cultivating types that are resistant to pests and herbicides. Up until this point, we have seen multiple examples of herbicide-resistant crops. With the advent of GR crops, a novel approach to efficient weed control, this technique has previously focused on in-crop glyphosate application. In order to make one type of sugarcane resistant to the herbicide glyphosate, the present study aimed to insert the cp4EPSPS gene into the plant via a ubiquitin promoter.

Our results show that TH is formed from the total sugar content at a rate 75% higher than that reported in previous research on sucrose isomerization, which only reached 40%. As a result of integrating promoters that target specific tissues in leaves and stems, this striking

variation is manifested. Evidently, vacuole-targeting signal peptides obtained from sweet potatoes are responsible for this novel behaviour; these peptides specifically target proteins produced and stored in the stem's vacuolar area. The enzymatic action of sucrose isomerase is prevented from reaching sucrose because vacuoles provide an unfavourable environment for the enzyme. As a result, additional TH might contribute to the overall SRP. The cytoplasmic sucrose is isomerized into TH-major and IM-minor by the activity of the gene ThSyGII triggered by coupled modified promoters. Compared to the early vegetative development from setts reported in open field conditions, the magnitude of SRP was lower during the preliminary growth of sugarcane lines in tiny pots under controlled greenhouse conditions. Closely spaced lines tend to prioritized biomass accumulation over improving SRP, according to observations made by experienced breeders. Sugarcane lines cultivated in wide fields with same climatic circumstances clearly did not differ much in SRP.

Also of great importance to researchers and impacting SRP were epigenetic modifications and differences in tissue culture settings. The present study provided a comprehensive review of the many facets of the sugarcane TH and SRP interaction. Total SRP increased by an astounding 77% when TH and IM production were increased to levels over 700 mM. When compared to the control plants at 16 months of age, genotypes with poor SRP production, such as SIP31 and SIP41, exhibited 24% higher SRP. Vigorous vegetative growth, enhanced photosynthetic efficiency, and phenotypic morphological traits were all hallmarks of the transgenic lines' rapid development. To fully understand gene stability across vegetative generations, additional field plot experiments are necessary to evaluate biomass gain precisely. The genus Saccharum and family Poaceae include sugarcane, a crucial crop for the food and beverage industries. It belongs to the monocotyledon family and is cultivated in nearly every region of the globe. It is possible to multiply sugarcane both sexually and asexually. With 600–700 genera and 10,000 species, the grass family is massive. Species of Saccharum include *S. officinarum, S. spontaneum, S. robustum, S. barberi,* and *S. sinense,* among others. Most people believed that sugarcane (Saccharum officinarum) originated as a monoploid plant from a combination of *Saccharum spontaneum, Erianthus asundinaceus,* and *Miscanthus sinensis*. Sugarcane has a very complicated genome since it is octaploid. From 80 to 120 chromosomes, the number varies. Due to its extremely polysomic and

heterozygous genome, sugarcane is typically propagated using cloning. The stalk internodes hold sucrose, which is mostly obtained from sugarcane. Chemicals, paints, plastics, synthetic materials, pharmaceuticals, fibres, detergents, pesticides, and a host of industrial goods such furfural, dextran, alcohol, paper, chipboard, confections, drinks, chemicals, paints, and sugar are among its most important uses. India, China, and Pakistan are the world's leading sugarcane producers, accounting for almost half of the total. A variety of sugar and byproducts made from sugarcane are grown in Pakistan, where the crop has a prominent position.

To make plants more resistant to herbicides, scientists have turned to genetic engineering. Sugarcane yields can be significantly increased by cultivating types that are resistant to pests and herbicides. Up until this point, we have seen multiple examples of herbicide-resistant crops. With the advent of GR crops, a novel approach to efficient weed control, this technique has previously focused on in-crop glyphosate application. In order to make one type of sugarcane resistant to the herbicide glyphosate, the present study aimed to insert the cp4EPSPS gene into the plant via a ubiquitin promoter. After the transformation was completed using a particle bombardment approach, molecular studies were performed to determine if the organism was transgenic and how it expressed the gene.

Sugarcane is a prime candidate for genetic engineering improvements due to several traits: A polysomy characterises sugarcane's genomic architecture, rendering conventional breeding for extreme changes fruitless. Efficient transformation systems are at hand, and vegetative propagation of transgenic lines can be done successfully and maintained for long, if not infinite, periods. Regulatory and public attitudes towards transgenic sugarcane are more favorable than those towards transgenic crops in general. The cp4EPSPS gene is one of many that contribute to glyphosate resistance. As a selective herbicide, glyphosate effectively suppresses broadleaf weeds while having little impact on the crop itself. Because the sugarcane genome is complicated and interacts with its environment in diverse ways, this traditional method of genetic modification has several drawbacks. When two or more inferior species are crossed, the desirable features of one or more of the other species can be unintentionally introduced.

Studies have shown that genetic modification, including transformation techniques, is often superior to traditional breeding approaches for introducing desirable traits into crops, such as glyphosate tolerance. As a result, genetic engineering is a potent method for designing germplasm with desirable features. Since freshly created transgenic lines may be kept for several years through vegetative propagation, the quick advancement in sugarcane genetic modification can be attributed to the establishment of effective transformation technologies. Particle bombardment has recently become a popular tool for sugarcane genetic engineering. Using this strategy to successfully change elite sugarcane genotypes has been documented in multiple papers.

Since the particle bombardment approach is not limited by tissue specificity, it can be used to change multiple genes more quickly and efficiently. These benefits led to the patenting of a method for producing GM sugarcane that expresses resistance to glyphosate by transforming the cp4EPSPS gene. The sugarcane lines utilized in this work were transformed by introducing the cp4EPSPS gene, which was expressed via the Ubi-1 maize ubiquitous promoter. Several earlier investigations found that the same promoter could efficiently endow sugarcane lines with expression. Studies on monocot species, such as rice, have demonstrated that the Ubi-1 promoter is very active during both stable and transient transformations. This promoter is essential for maize development. One example is the Ubi-1 promoter, originally from maize, which, when introduced into various sugarcane lines, showed significantly higher expression levels than Act1 and CaMV 35S, two other popular promoters. While chromosomal rearrangement and foreign gene integration typically result in non-homologous recombination, DNA rearrangement might impact expression after transformation. Because of this, there is no guarantee that the host's gene expression will be consistent. There are a lot of factors that determine gene expression, including the copy quantity of the inserted gene, the location of the foreign DNA integration site relative to the host genome's regulatory elements, and the type of promoter that drives the gene. All of these moving parts highlight how difficult sugarcane genetic engineering is, and how important it is to optimise transformation techniques in order to express integrated characteristics as intended. With the world's sugar consumption on the rise, boosting sugarcane output is essential. Consequently, there is a need for innovation, such as the use of genetic modification

technologies to enhance herbicide-resistant cultivars, due to low average yields in countries like Pakistan. Other transformation methods, including as particle bombardment, and the use of efficient promoters, such Ubi-1, could further improve this in sugarcane with respect to weed competitiveness. Regions dependent on sugarcane will benefit from increased food security and economic stability thanks to new developments in the field of transformation, which is also the future of sugarcane production. Using polymerase chain reaction (PCR) with gene-specific primers, as previously mentioned, the potential transgenic cotton plants were confirmed. In order to get both quantitative and qualitative estimates, dot blot analysis was also performed on GTGene after PCR. It was believed that the gene was present because the blot showed an expression as a bright reddish color. Dipstick Immuno-strip tests (EnviroLogix ®) were then used to determine the presence of the necessary proteins in the transgenic cotton plants. In line with prior research, the immunostrip test results consistently demonstrated positive results for GTG, Cry1Ac, and Cry2A across all converted plants. The presence and expression of GTGene in the transformed lines were confirmed by the bands that emerged from this assay.

After cotton, sugarcane is Pakistan's most important cash crop, accounting for 3.6% of value-added agriculture and 0.8% of GDP. Sugarcane is mostly harvested for its sugar, which is then processed into a number of industrial products including alcohol, dextrans, furfural, and stored internodes. Some natural medicinal formulations include sugarcane in one way or another. It finds use in paper, animal feed, and food production as well as in many industrial and agricultural by-products. In 2011 and 2012, Pakistan's average sugarcane output was at 58.0 tonnes per hectare, which was lower than the 65 tonnes per hectare average worldwide. The yield of sugarcane is frequently significantly reduced by undesirable plant species. Weeds are one of these issues, and they're mostly to blame for the decline in sugarcane yield and quality. Reduced yields are the end result of all the rivalry for water, nutrients, and light that occurs during the growth season. According to studies conducted in Pakistan, weed competition is responsible for a sugarcane output drop of from 15% to 30%. Worldwide, weed pressure on sugarcane output reduction is between 12 and 72 percent. A large amount of labor is required by the majority of the conventional weed management strategies for sugarcane fields. Plant uprooting and potential worker injuries from mechanical weed

eradication are only two examples of how many of these methods are physically harmful to the soil. Most weed species have deep and wide roots, which makes it difficult to completely remove those using conventional methods. What's more, most of them have the potential to regenerate quickly. Weed control in agricultural systems has undergone a sea change since the advent of herbicide-resistant crops. Herbicide tolerance can be introduced into the Saccharum gene pool by a combination of classical breeding and genetic engineering. Traditional breeding methods are inefficient when used to sugarcane's wild relatives because they lack the herbicide-tolerant genes. Transgenic technology has the potential to increase yields since it can be used to create herbicide-resistant types at a lower cost than traditional weed management methods. Several crops have been genetically engineered to withstand herbicides thus far. Roundup Ready soybeans, which include a variant of the EPSPS gene that gives resistance to glyphosate and is derived from the bacteria *Agrobacterium tumefaciens*, are among the most extensively grown genetically engineered crops. Current sugarcane crop management practices include the use of Organophosphorus herbicides, the most prevalent of which being Roundup.

Glyphosate as a broad-spectrum pesticide

A broad-spectrum pesticide that translocate simplistically to the meristems of growing plants, glyphosate is the active component of Round up. In comparison to other pesticides, glyphosate is safer to use, has low residual levels in soil and organisms, and has little to no influence on the environment. The emergence of weed resistance to glyphosate-based pesticides has recently come to light. Multiple studies have shown that glyphosate becomes immobile inside the soil matrix after entering a plant's root system and being absorbed. Once there, it is released into the rhizosphere, the soil zone around the root. This kind of impact has the potential to upset ecological equilibrium by changing the makeup of soil and root microbes. In addition, some studies have suggested that rainstorm events following glyphosate treatment can cause up to 1-2 percent of the applied amount to runoff. The issue of environmental persistence is once again brought up by this. Research in the lab has shown that even trace amounts of glyphosate can cause birth abnormalities in birds and amphibians. So, beyond its function as herbicide, glyphosate use has consequences for

environmental safety and impacts on non-target creatures. Making progress towards transgenic sugarcane types with increased herbicide resistance ranks high among the most significant achievements of contemporary agriculture. While reducing the negative effects on the environment caused by traditional weed control methods, this novel genetic engineering strategy can eliminate weeds altogether and increase sugarcane output potential. As the world's demand for sugar continues to rise, the integration of biotechnology into the production cycle will be crucial in addressing food security and economic concerns in this vital agricultural industry. Glyphosate is a pesticide that kills plants, especially weeds, by blocking an enzyme called chloroplast-localized EPSP synthase 5-enolpyruvylshikimate-3-phosphate synthase. Transformed plants that have had their EPSPS gene overexpressed in their cells have shown signs of glyphosate tolerance, meaning they can continue to grow even when exposed to the herbicide. Differences in the reported key pathways for conferring glyphosate tolerance in plants include the following: Overexpression of EPSPS and an enzyme variation that is resistant to herbicides are two of the mechanisms. An efficient transformation system is crucial for sugarcane genetic engineering, which is associated with the callus's embryogenic capacity and its ability to regenerate into whole plants.

In order to improve these sugarcane varieties by genetic engineering, the present study set out to establish herbicide resistance in a few chosen elite cultivars indigenous to Pakistan. Transgenic crops harboring non-glyphosate herbicides such as 2,4-D, Dicamba, HPPD inhibitors, and the bar gene are among the herbicide-tolerant crops that have been created thus far. Although these transgenes were designed to work with particular herbicides, glyphosate-tolerant crops were created to offer a wider range of weed control options, not limited to just one herbicide. Soybean, corn, cotton, canola, sugar beetroot, Polish canola, wheat and creeping bent grass are the nine crops that have been designed to withstand glyphosate thus far. Except for creeping bent grass and wheat, every single one of these has been farmed for commercial purposes. The study that has been detailed here is the initial effort to create sugarcane that is resistant to glyphosate. This is because Pakistan has the fourth-highest area under sugarcane cultivation globally, thanks to its favorable agro climatic condition. The yields per unit area are not very high. Establishing a consistent and repeatable process of transformation has long been a significant challenge in sugarcane genetic engineering due to the crop's

inherent resistance to change. The goal of this research was to develop transgenic plants with strong glyphosate resistance and a method for sugarcane callus regeneration that is both systematic and efficient.

The genetic alteration of sugarcane and the establishment of an effective transformation system are both reliant on the optimization of friable, embryogenic calli. We cultivated friable and embryogenic calli in all four cultivars using 2,4-D media; to increase the calli's embryogenic potential, we added casein. Additional research has demonstrated that 2,4-D is necessary for sugarcane to produce both friable and embryogenic calli. The emphasis is on microshoots regeneration and roots because our investigations aimed at producing microshoots from callus masses instead of trying somatic embryogenesis. The four sugarcane types showed distinct genotypic heterogeneity in terms of the amount of callogenesis and regeneration. Regeneration efficiency was also shown to decrease when the content of 2,4-D in the callus media was increased to greater than 4 mg/L. The ability to regenerate was completely lost at greater concentrations. This is in line with previous research that found a decrease in fresh mass of calli when 2,4-D rates were high.

When testing for glyphosate resistance, the general elite varieties CPF-234, CPF-213, HSF-240, and CPF-246 were utilized for gene transformation. The tolerance levels of the transgenic plants to glyphosate sprays were much higher than those of the non-transgenic control plants. To assess the plants' tolerance, two different concentrations of glyphosate were sprayed: 800 mL and 1100 mL per 0.404 hectares. Although some transgenic plants did experience slight leaf browning after the initial glyphosate spray, the majority of transgenic plants were unaffected. In contrast, weeds and non-transgenic plants died off after the first application. Another application of glyphosate was performed at a rate of 1100 mL to further examine the tolerance of this herbicide in the transgenic plants. The non-transformed control plants of the sugarcane species developed necrotic signs and died as a result of this application. The amount of transgene-expressed protein varied among transgenic plants, nevertheless. Treatment with 1100 mL of glyphosate per 80 L resulted in browning and the death of plants with OD of trans-protein below 1.0, while plants with OD of trans-protein above 1.0 survived.

Based on these findings, it seems that transgenic sugarcane plants do, in fact, exhibit glyphosate tolerance that is positively correlated with transgene expression levels; thus, it follows that higher transgene expression levels result in more tolerance. Variation in transgenic expression may be influenced by the position of transgene incorporation into the plant genome. Discolouration and light browning were the first signs within the necrotic tissues of transgenic sugarcane plants treated with glyphosate. After experiencing these symptoms for roughly 7 to 10 days, the plants' original appearance was restored. Because glyphosate blocks the enzyme EPSPS, which makes the aromatic amino acids tyrosine, phenylalanine, and tryptophan, this is the result. Sugarcane plants have their native EPSPS enzyme inhibited when sprayed with glyphosate. Plants that carry the glyphosate-tolerant transgene are able to recover from the harmful effects of glyphosate because the modified EPSPS sequence helps them to resist the inhibitory activity of the herbicide. In this way, the transgenic sugarcane plants are able to overcome the harmful impacts of glyphosate stress and begin to thrive again. Transforming sugarcane to be glyphosate-tolerant is a huge step forward for agricultural biotechnology and a great way to keep weeds at bay when growing sugarcane. Increased sugarcane yield and sustainability, reduced weed competition losses, and improved food security in areas reliant on this cash crop are all possible outcomes of breeding these genetically modified varieties. While there was some variance in transformation efficiency across the sugarcane types evaluated, this investigation showed that all of the kinds exhibited repeatable transformation processes. One of the biggest challenges in growing sugarcane is eradicating invasive plant species, which can drastically lower harvest yields. Attempts to create sugarcane cultivars that are resistant to herbicides will greatly benefit agricultural output, as stated above. In addition, biotechnologists can increase sugarcane resistance to infestation, fungal pathogens, insect attacks, and related illnesses by developing an effective transformation system. Past studies on wheat resistant to glyphosate have shown that this herbicide can both prevent and treat rust infections caused by the fungi *Puccinia striiformis f. sp. tritici* and *Puccinia triticina*. Wheat rust was effectively controlled at all plant growth stages examined in growth chamber experiments when applied at the recommended rates for weed

management. Field trials showed that the same cultivar maintained full tolerance to glyphosate, with no plant mortality even at the greatest glyphosate application rate, and weed control was effective in these crops. In a similar vein, researchers have used the same concept to create sugarcane types that are resistant to herbicides, demonstrating the efficacy of transgenic methods in agricultural biotechnology. After conducting thorough studies on the agronomic performance and gene expression stability of transgenic herbicide-resistant sugarcane, researchers determined that glyphosate-tolerant transgenes are effective at concentration that enable glyphosate applications to control plants and weeds.

One of the main macronutrients needed for plant growth and development is phosphorus, among all the other general elements in plants. There is a physical limit to how much phosphorus plants may absorb from soils due to the element's solubility and fixing characteristics. Soil solutions often only contain trace amounts of phosphorus—a few parts per million, or less than one percent—that plants can absorb. The majority of the phosphorus that plants absorb through their roots is in the forms of H2PO4 and HPO4^2-, which vary according to the soil pH. Soil bacteria called phosphate-solubilizing bacteria (PSB) are responsible for loosening insoluble phosphorus molecules, allowing plant roots to absorb the metabolites. From one soil sample to the next, PSB populations range from one percent to fifty percent of the overall soil microbial population, demonstrating their pervasiveness in nature. Bacteria are metabolically more active in the rhizosphere, where they also tend to congregate more than in other isolation sources. Their rhizosphere-dwelling natural occurrence was documented over a hundred years ago. It is hardly surprising that these PSB have become so vital for soil, considering how old they are. This means that among the most effective phosphate-solubilizing bacteria, the genera Pseudomonas, Bacillus, Rhizobium, and Enterobacter stand out as the most significant. One of the effective ways that PSB solubilises phosphates is by producing organic acids and acidifying the media. An effective increase in plant availability of phosphorus is caused by these organic acids, which promote the conversion of tricalcium phosphate into di- and mono-basic phosphates. The amount and variety of products made by various creatures varies greatly. Of all the organic acids that dissolve phosphate, two of the most typically formed are glutonic acid and 2-ketogluconic acid. It has been claimed that this solubilization process

also involves other organic acids as acetic, citric, lactic, propionic, glycolic, oxalic, malonic, succinic, fumaric, and tartaric acids.

Both plants and certain microbes manufacture indoleacetic acid, a fundamental plant hormone. By means of distinct importers and efflux pumps, IAA is transported throughout the plant body and plays a role in the growth of both roots and shoots. One of the most important growth regulators for improved growth and root development is IAA, and PGPRs aid the plant in synthesizing this compound. This is a critical metric for evaluating PGPR's ability to stimulate plant growth. Worldwide crop output is under jeopardy due to the rise in food security issues caused by pathogenic microorganisms' impact on yield loss. Agrochemicals are a typical tool for crop protection in contemporary farming, protecting crops from various diseases. However, there are major consequences for non-target creatures due to the increased use of these pesticides. So, to lessen the impact of chemicals on agriculture, the use of biocontrol agents, such as beneficial bacteria, has been touted as a viable and appealing option. Rumour has it that rhizospheric microbes have a direct hand in plant morphology and produce large quantities of growth-promoting chemicals. Here we offer one of the practical applications of correlation analysis: selecting top performers by gauging the strength of relationships between different criteria. As a result, worldwide worries about food safety and environmental sustainability have prompted calls to replace synthetic chemicals with PGPR. To improve crop development, seed germination, and overall yield, numerous PGPR formulations have been created; a few of these have even reached commercialization.

Using sugarcane and rice rhizospheric soil, this work isolates and characterizes possible PSB. In order to determine whether there would be any benefits to using the separated strains in combination, we conducted a thorough correlation analysis among them. One of the most important elements for plant growth is phosphorus. The conversion of phosphorus from chemical fertilizers into insoluble calcium or magnesium salts in the soil renders a significant portion of this element unavailable to plants. Soil microbes play a crucial role in making phosphates available to plant roots by breaking down insoluble phosphorus molecules into soluble ones. Because of the many positive ways in which PSBs contribute to soil ecosystems, they have an impact on soil health both directly and indirectly. The breakdown of organic

matter, the mineralization of nutrients, and the fixing of nitrogen are all mediated by these creatures. The current investigation evaluated fifteen PSB isolates for phosphate-solubilizing characteristics. Confirming previous findings about the efficacy of NBRIP medium for solubilization activity, all of the isolates were shown to solubilize phosphorous when cultured on Pikovskaya and NBRIP media. Consistent with other research, our results showed that the PSB isolates effectively dissolved tricalcium phosphate. The PSB isolates were identified and characterized using a combination of morphological, biochemical, and molecular approaches in this investigation. Gramme staining, oxidase, catalase, and QTS-24 assays were utilized in addition to morphological examinations that included colony and cell morphology.

Molecular Identification and Phosphate-Solubilizing Efficiency of PSB Isolates

To identify PSB molecularly, 16S rRNA gene sequencing was employed. The ability to solubilize phosphate was demonstrated by all isolates following three days of incubation. This might be because the media pH dropped as a consequence of the PSB growing healthily. It was also confirmed that the PSB isolates could produce IAA. There have been reports of strains that produce IAA, which is associated with substantial impacts on plant development and growth, being isolated from the rhizosphere of several crops. One reason PSB strains could be a good PGPR is that they can produce IAA and solubilize phosphorous in media at the same time. The dual culture method on potato dextrose agar plates demonstrated that certain PSB strains were hostile to the fungal pathogen *Fusarium*. The multifaceted positive properties of the microorganisms are further enhanced by the fact that certain strains of the genus Pseudomonas of PSB exhibit extraordinary antagonism against pathogenic fungi such as *Rhizoctonia solani.* According to the results of the current study, there is a strong and substantial correlation between the percentage of phosphate solubilization for a large number of PSB isolates, including R-1, R-2, R-3, SC-01, SC-03, SC-05, SC-07, SC-20, SC-19, SC-17, and SC-22. A link of this kind would suggest that different isolates, when combined, might significantly increase crop production and phosphate solubilizing rate.

The plant's ability to use and absorb large quantities of phosphates should be enhanced by selecting PSB isolates. Isolates of P-solubilizing *Rhizobacteria* (R-1, R-3, SC-01, SC-03, SC-07, SC-09, SC-13, SC-19, SC-17, SC-20, and SC-22) exhibited strong correlations with one another and with the antifungal activity against Fusarium, as measured by the stimulation of growth inhibition zones. Based on these robust connections, it is possible that combining these PSB isolates would have a greater antifungal effect than using them alone. In addition to improving crop tolerance against biotic stresses, using herbicide-tolerant sugarcane cultivars offers an indirect strategic advantage for weed management. Beyond this, PSB's significance as promising future crop inputs in sustainable agriculture has been balanced by their functions in phosphorus solubilization and plant development. To further understand how these innovations function, how to apply them in the field, and what impact they could have on soil health and agricultural yields in the long run, more research is needed. By ensuring the development of synergy between genetic engineering and microbial application, agriculture can ultimately confront modern difficulties. This will lead to better food security and environmental sustainability. Grown in 58 different nations, sugarcane is a perennial tropical crop and one of the world's most important cash crops. About 26.9 million hectares are devoted to cane agriculture on a global scale. Nearly eighty percent of the world's sugar, primarily in the form of sucrose, comes from this genus *Saccharum*. The stalks store sucrose in their internodes, and sugar mills process almost two billion metric tonnes of stalks every year to produce juice that contains sucrose. This source accounts for almost two-thirds of the sugar produced globally. Fibre, biofuels, chemicals, and industrial commodities such as alcohol, furfural, dextran, particleboard, paper, drinks, candies, plastic, paints, synthetics, pharmaceuticals, industrial enzymes, pesticides, and detergents are all made from sugarcane, a complex feedstock crop. Sugarcane accounts for about 0.7% of Pakistan's GDP and 3.4% of the value-added agricultural sector, out of a total of 1.2171 million hectares in cultivation. Pakistan is now one of the world's top five sugarcane-producing nations, with an increased total output of 73.6 million metric tonnes. As a major export commodity for Pakistan and a source of income for more than nine million people, the crop is critically essential to the country's economy. It is the backbone of the country's second-largest agricultural sector, supplying 84 sugar mills with raw materials. These mills often manufacture sugar domestically and sell it to Central Asian nations like Tajikistan and Afghanistan.

When it comes to the availability of sugarcane, the sugar sector is highly dependent on the country's sugar producing capacity.

Tissue Culture and Regeneration Techniques for Transgenic Sugarcane

The calli effectively transformed into microshoots, and subsequent to their multiplication, the commencement of roots was noted. The inherent genotypic heterogeneity for in vitro tissue culture performance regarding callogenesis and regeneration resulted in a significantly diverse response among these four types. Additionally, it was noted that the regeneration efficiency was negatively impacted by 2,4-D concentrations in the callus induction medium exceeding 4 mg/L. The regeneration ability of these calli was really rendered null and void at greater 2,4-D concentrations. Previous research has shown that calli biomasses decreased in response to increased 2,4-D concentrations in the media, therefore this follows suit. Apart from conducting glyphosate field spray experiments, the percentage of surviving and regenerated calli on selection media were used to measure the four kinds' tissue culture responses and transformation efficiency. Based on the results of multi-stage screening, CPF-246 was determined to be the most suitable variety for future advanced field investigations. With a callus induction rate of 90%, a regeneration rate of 99%, and a micro-shoot multiplication rate of 100%, CPF-246 demonstrated excellent performance in tissue culture investigations. According to previous research, different types of were distinguished by the way they responded to genetic manipulation in terms of regeneration.

Insecticidal genes (Cry1Ac and Cry2A) and glyphosate-tolerant genes (GTG) engineered in plants have only improved upon conventional breeding methods. Sugarcane that has been transgenic and engineered to express Bt genes is more resistant to sucking cane borer insects. So far, over a hundred Bt genes have been sequenced, revealing notable variations in biochemical features and amino acid sequences. Researchers found that a combination of Cry1Ac and Cry2A was significantly more efficient than each toxin alone against borer insects and all other species of Lepidoptera. One example of gene pyramiding, the approach to develop numerous features into one variety, is the introduction of both the Bt genes Cry1Ac and Cry2A on one variety. Among the many pesticides used today, glyphosate stands out as a

non-selective herbicide that is incredibly popular. This is due to the fact that glyphosate inhibits the growth of numerous plant species, including the majority of weeds and herbs. The shikimate metabolic route is where glyphosate's herbicidal effects are felt. This pathway prevents plants from making tyrosine, tryptophan, and phenylalanine, three essential amino acids. Over the years, a number of herbicide-resistant crop varieties have been developed in rapid succession. Among these are bar transgenes, HPPD inhibitors, 2.4-D, and Dicamba, which are transgenics that impart tolerance to herbicides other than glyphosate.

Optimizing Auxin and Cytokinin Combinations

Different kinds of callus cultivated on different media have been reported in other papers to have diverse colors and textures. The calluses collected were of two varieties: one was loose and friable and embryogenic, while the other was compact and white and nodular and embryogenic as well. Both regenerative and non-regenerative calluses were noted. The fact that some of the little calli were white in this context suggests that they may be able to undergo somatic embryogenesis and subsequent plantlet regeneration, both of which have embryogenic potential. The presence of somatic embryoids, which could be detected prior to transferring the calli to an embryogenic media, caused the callus to appear white. In order to determine the impact of various hormonal combinations on embryogenesis, calli that were five weeks old were chosen and placed on embryogenic media that had been prepared with various combinations of auxins and cytokinins. The SC3 variety was primarily used for selection because of its ability to produce compact and embryogenic calli, which helped in somatic embryoid development and plantlet regeneration.

The 2,4-D// BAP and IAA//BAP hormone combinations, with a 1 millennial each and a 2 mg/L concentration of each hormone, produced some of the most promising outcomes of the several that were tested. White, granular somatic embryoids were more likely to form within a week or two of being transferred to the embryogenic media when these various combinations were used. It is worth noting that within three weeks of being exposed to the hormone combinations, little plantlets were also seen growing back from the

nodular structures themselves. The optimal values were 2+2 mg/L for the 2,4-D with BAP combination and 2+3 mg/L for the IAA with BAP combination; both were higher than the lower amounts. Although different kinds grew at different rates, they all maintained several plantlets on callus tissues. In general, it seems that a high auxin plus cytokinin combination promotes callus proliferation and embryo induction, whereas a high cytokinin concentration is better for plant regeneration. The use of auxin and cytokinin in indirect somatic embryogenesis was demonstrated to be successful. According to another paper, the interaction between auxins and cytokinins had a substantial impact on somatic embryogenesis and plant regeneration. The best results were obtained in sugarcane types BL-4 and CP 77,400 when the ratio of 2,4-D and BAP was 1+0.25 mg/L. it was found that a 2+1 mg/L concentration of 2,4-D and BAP had the greatest impact on sugarcane variety Co 671's somatic embryogenesis and plant regeneration, they replicated these findings.

Research has shown that high 2,4-D concentrations, when applied to callus tissues over an extended period of time, promote the development of somatic embryos. When embryo genic callus segments were cultivated in medium supplemented with a mixture of auxin-cytokinin, namely 0.53 mg/L of 2,4-D and 5% coconut milk, Ho and Vasil found that a considerable number of somatic embryos were formed. The embryos were generated from young leaves. Furthermore, embryogenic callus was generated after 3.0 mg/L of 2,4-D was applied, indicating that a rather large dosage of auxin is required to commence dedifferentiation and the production of embryogenic cells. Further, they claimed that it is crucial to keep auxin levels appropriate at later stages of embryogenesis. From a broader viewpoint on somatic embryogenesis, research indicates that lower 2,4-D concentrations (1.0 or 0.5 mg/L) do not promote effective somatic embryogenesis. In contrast, concentrations ranging from 1.5 to 3.0 mg/L were linked to the attainment of up to 80% somatic embryogenesis. This further supports the idea that in order to successfully form embryoid and regenerate plants, ideal hormonal conditions should be set up. The current research adds to our understanding of sugarcane somatic embryogenesis and highlights the crucial role of auxin/cytokinin in inducing callus and plantlets. The goal of all the research is to find better ways to produce germplasm that is free of diseases so that sugarcane growing may become more productive and of higher quality. Everyone's dream is to optimise this

process. Therefore, further research on improving the efficiency of somatic embryogenesis and plant regeneration procedures in sugarcane and other crop species could greatly benefit from this current study.

Pest and Insect Resistance in Sugarcane Cultivation

Crops modified to carry the cp4EPSPS gene are resistant to more than just one type of herbicide, in contrast to these transgenic crops, which were only resistant to a handful of them. Because of this broader resistance, less chemical inputs are needed to control weed populations. Utilizing genetic engineering techniques enables the creation of sugarcane varieties that are not only resistant to pests and herbicides, but also have the potential to produce more than current commercial kinds. This leads to more productive and environmentally friendly agricultural practices. One promising strategy for tackling the complicated problems encountered by sugarcane growers is gene pyramiding, which involves combining multiple features into a single variety. In order to enhance food security on a worldwide scale, this technique employs participatory methods to create sugarcane cultivars that are suitable for various agro-ecologies, while also providing economic advantages to farmers. One of the world's most valuable cash crops, sugarcane is a tropical perennial. It is cultivated in 58 distinct nations. About 26.9 million hectares are devoted to cane agriculture on a global scale. This genus Saccharum provides the majority of the world's sugar, sucrose, accounting for almost 80% of the market demand. The stalks store sucrose in their internodes, and sugar mills process almost two billion metric tonnes of stalks every year to produce juice that contains sucrose. This source accounts for almost two-thirds of the sugar produced globally. Sugarcane is a multi-use crop that produces sugar as well as fibre, biofuels, chemicals, and various industrial commodities including alcohol, furfural, dextran, particleboard, paper beverages, artificial sweeteners, paints, synthetics, pharmaceuticals, industrial enzymes, pesticides, and detergents. With 1.2171 million hectares under sugarcane, Pakistan's agricultural industry accounts for 3.4% of the value-added agricultural output, or around 0.7% of GDP. Given the current trajectory, Pakistan is now one of the world's top five sugarcane producers, with an overall output of 73.6 million metric tonnes, thanks to all of the country's joint efforts. Producing over nine million jobs and playing a significant role

in Pakistan's export economy, the crop is also of utmost importance to the country's economy. It is the backbone of the country's second-largest agricultural sector, supplying 84 sugar mills with raw materials. These mills often manufacture sugar domestically and sell it to Central Asian nations like Tajikistan and Afghanistan. When it comes to the availability of sugarcane, the sugar sector is highly dependent on the country's sugar producing capacity.

Worldwide, pesticide use is hastening environmental deterioration and endangering human health. Pests and illnesses are responsible for almost 37% of the yield loss in global agricultural production, with insects accounting for around 13% of that loss. Any bug that feeds on plants, whether it's a crop, stored product, or decorative plant, falls into this category. There are 61 identified insect species in Pakistan, out of a total of 1300 that are known to attack sugarcane crops globally.*Chilo infuscatellus* and Chilo auricilius are the most common species of borer in Pakistan. These borers are known to cause stem losses of 15% to 36%.Scirpophaga novella and Scirpophaga excerptalis are other significant borers recognized by both literature and farmers; infestations caused by these species can result in losses of 10% to 15%. Crop infestations by the root borer Emmalocera depressella can reach 10% to 20%.

One pest that causes around 20% damage is *Acigona steniella*, more often known as Gurdaspur borer. The sugarcane leafhopper, *Pyrilla perpusilla*, can cause a loss of up to 25%. The devastating *Aleurolobus barodensis* whitefly can infest 80 percent of a plant's leaves. Yield loss can reportedly be anything from fifteen percent to twenty-five percent. Sugarcane is also subject to other minor pests such as mealybugs (*Saccharicoccus sacchari*) and black bugs (*Cavelerious excavatus*). The increasing and heavy use of pesticides and insecticides is a yearly trend that growers are responding to by attempting to prevent losses caused by these types of insect assaults. Transgenic plants are protected from significant *Lepidopteran*, Dipteran, and Coleopteran pests by crystalline proteins produced by using the cry genes from *Bacillus thuringiensis.*

Currently, there are more Cry gene variants found in Bt crops that have proven to be highly effective in controlling *Lepidopteran* insects. These types include Cry1Ab, Cry1Ac, Cry1Ac + Cry1F, Cry2A, and Cry1Ac+ Cry2A. Compared to non-transgenic crops, transgenic Bt crops may

require five to twelve pesticide applications less often throughout the growing season. From a chemical perspective, glyphosate is also known as N-phosphonomethyl glycine. It is an effective herbicide for controlling weeds since it is broad-spectrum and very active. It works by blocking an enzyme called EPSPS, which is involved in the production of aromatic amino acids and plays a critical role in plants' shikimic acid pathway. The transgenic plants are able to maintain their tolerance to glyphosate because they include genes that encode a soil bacterium form of the EPSPS enzyme. Along with its low toxicity to humans and other non-target creatures, Glyphosate's distinctive non-selective mechanism of action is a major factor in its widespread use. Consequently, "better self-sufficiency" was stated as the overarching objective of this initiative, along with "meeting the demands of an ever-growing world population" and reducing the prevalence of cane borers and weeds. Despite a number of obstacles, including cane borer infestations, weed competition, drought stress, and virus infections, the end goal of sugarcane cultivation is still high yield. To manage cane borers and weeds, the present inquiry was established by genetically transforming sugarcane with insect-resistant codon-optimized genes [CEMB-Cry1Ac + CEMB-Cry2A] and the glyphosate tolerant gene [CEMB-GTGene].

Pakistan has ideal Agroclimatic conditions for sugarcane, however the country ranks fifteenth in sugar production while having the fifth-largest land area, suggesting a low yield per unit area. Primarily concerned with monocot species, this research seeks to develop a reliable and repeatable transformation technique for regenerating transgenic plants while simultaneously introducing resistance and tolerance to cane borers and the herbicide glyphosate. The tissue culture trials utilized four local elite varieties: CPF-246, HSF-240, CPF-213, and CPF-234. The immature leaves are utilized as explants for callus induction because, according to multiple studies, they are great for producing embryogenic callus, a crucial component of sugarcane genetic transformation. In this investigation, embryogenic calli were generated from each of the four types using a callus formation media that included 2,4-D. Casein, which increases sugarcane calli embryogenic competency, was added to the medium to further enrich it.

We effectively turned the generated calli into microshoots, and once the microshoots multiplied, we witnessed rooting initiation. Because of

the underlying genetic heterogeneity in the four kinds, the response varied greatly in terms of how well they performed in vitro tissue cultures for callogenesis and regeneration. A negative impact on regeneration efficiency was noted when the concentration of 2,4-D in the callus induction media exceeded 4 mg/L. The regeneration ability of these calli was really rendered null and void at greater 2,4-D concentrations. This is in line with other research that found lower calli biomasses when 2,4-D concentrations were higher in the media. The most suitable variety to proceed with generation advance field experiments was determined to be CPF-246 following this multi-tier screening. In addition to conducting glyphosate field spray experiments, the four types' transformation efficiency and the percentage of calli and regenerated calli that survived on selection media were assessed. An impressive 90% callus induction rate, 99% regeneration rate, and 100% microshoot multiplication rate were achieved by the high-performing variety CPF-246. Prior research focused on the types examined for their regeneration response when subjected to genetic engineering. The benefits of glyphosate-tolerant genes, such as GTG, and insecticidal genes, such as Cry1Ac and Cry2A, introduced into plants through genetic modification outweighed those of conventional breeding alone. The transgenic sugarcane showed improved resistance against sucking cane borer insects due to increased expression of Bt genes. So far, over a hundred Bt genes have undergone sequencing, revealing significant variations in metabolic features and amino acid sequences. The research shows that a combination of the toxins Cry1Ac and Cry2A is far more powerful against *Lepidopteran* insects, including borer insects. One example of gene pyramiding, the approach to develop numerous features into one variety, is the introduction of both the Bt genes Cry1Ac and Cry2A on one variety.

Glyphosate is a non-selective herbicide that is currently among the most utilized pesticides. Reason being, glyphosate inhibits the growth of numerous plant species, including the majority of weeds and herbs. The shikimate metabolic pathway is the mechanism by which glyphosate kills plants. Tyrosine, tryptophan, and phenylalanine are three amino acids that plants need for survival, but their synthesis is blocked by it. This compound is called 5-enolpyruvyl-3-phosphoshikimate, or EPSPS. Many herbicide-resistant crops have emerged in recent generations. 2.4-D, Dicamba, HPPD inhibitors, bar transgenes, and other non-glyphosate herbicide-tolerant transgenics

are part of this group. While these transgenic crops were only resistant to a small subset of herbicides, crops engineered to carry the cp4EPSPS gene would be resistant to all of them. This would allow for more leeway in weed management and less reliance on chemical inputs. New sugarcane types, made possible by genetic engineering, are not only more productive and environmentally friendly than current commercial kinds, but they are also more resistant to pests and herbicides. One promising strategy for tackling the complicated problems encountered by sugarcane growers is gene pyramiding, which involves combining multiple features into a single variety. This strategy aims to improve food security on a worldwide scale by promoting economic benefit to farmers through the use of participatory approaches in sugarcane varietal development across varied agro-ecologies. Soybean, wheat, canola, corn, sugar beetroot, creeping bentgrass, cotton and Polish canola are among the glypohosate-resistant crops that have been commercially released thus far. This biotechnology has made it possible for farmers to control weed populations while maintaining competitive crop yields. One of our earlier studies, involved transforming sugarcane using modified sequences from CEMB for Bt genes (CEMB-Cry1Ac and CEMB-Cry2A) and a glyphosate-tolerant gene (cp4EPSPS). Lacklustre expression levels were the consequence of early attempts to produce Bt toxins using nuclear-encoded chimaera genes. The poor expression was thought to be caused in part by the AT-richness of the Bt genes compared to plant genes. Basically, there are a number of potential causes for this, including poor codon utilization, aberrant mRNA splicing, unstable mRNA, or early transcription termination.

To circumvent these problems, the synthetic genes that encode Bt toxin were engineered, built, and cloned in a manner that reduced the prevalence of these unwanted traits. At last, and most critically, this improved the plant's expressed copy of these transgenes. Toxin expression reached 0.8% of total leaf protein, insect borer resistance increased dramatically, and toxin stability was improved through the inclusion of several promoters and other regulatory components. We developed and successfully transformed three synthetic genes in sugarcane—CEMB-Cry1Ac + CEMB-Cry2A and CEMB-GTGene—with codon optimization. This synthetic gene set was engineered using the Ubiquitin-1 promoter found in maize.

To achieve high levels of protein production, the transgenes were organized into simple and minimal plasmid cassettes. Using either the CAMV35S or maize Ubiquitin-1 promoters, the expression levels of glyphosate-tolerant genes were compared to those of the Bt gene. The results showed that the expression of interest was around twice as high as the CAMV35S promoter, according to the analysis. Therefore, the Ubiquitin promoter was used to increase the expression of transgenes in maize with the goal of making the crop more resistant to cane borers and glyphosate herbicide. Transformed calli were used to regenerate a large number of transgenic sugarcane plants using the plant expression constructs pCEMB-SGTG for the glyphosate-tolerant gene CEMB-GTGene and pCEMB-SC12 for the CEMB-Cry1Ac + CEMB-Cry2A genes. In this research, we confirm that the three transgenes are stable and pass them on from one vegetative generation (V0) to the next (V1, and V2). The results showed that 81 out of 100 converted calli that were exposed to 50 mg/L of kanamycin underwent selection and ultimately survived. In addition, regeneration was achieved by 48% of the converted calli when placed on a dual selection medium that contained both glyphosate and kanamycin. Following optimization of the glyphosate spray assay, a transformation efficiency of 1.5% was achieved with 15 out of 1,000 resistant multiplied plants from variety CPF-246 testing positive for CEMB-Cry1Ac, CEMB-Cry2A, and CEMB-GTGene. There is evidence that the transformation efficiencies of monocots fall somewhere between one percent and five percent. When a wide variety of plasmids were used for transformation, the co-integration of the two genes might reach 85%. Research on sugarcane has shown transformation efficiencies between 0.8% to 4.8%.

There are around 12–16% soluble sugars, 11–16% fibre, and about 2%–3% water in the mature stem of *Saccharum officinarum L.,* among other ingredients. Carbohydrates, proteins, and minerals abound in sugarcane juice. A few examples are calcium, iron, potassium, salt, zinc, thiamine, and riboflavin. It also contains antioxidants such flavonoids and phenolic compounds. If you're looking to enhance your immune system and ward off diseases like cancer and cardiovascular ailments, this composition is for you. This is where the use of genetic modification techniques to enhance sugarcane's many properties has recently emerged as a major focus of research. Thanks to biotechnology, pest and herbicide resistance characteristics may now be reliably introduced into crops and expressed in their offspring. In the event that genetically modified organisms (GMOs) are successfully

introduced into the food chain, they will likely impact crop breeding practices worldwide, particularly in relation to the development of more insect-resistant varieties. An growth in the number of biotech crops cultivated globally is evidence of the well-documented momentum of biotechnology in agriculture. A small number of crops have achieved unprecedented levels of global prominence due to a genetic combination of insect resistance and herbicide tolerance.

One of Pakistan's most important agricultural products is sugarcane, widely recognized as one of the world's most valuable cash crops. Many agro-based companies rely on it as a source of raw materials. Sugarcane is not only a big moneymaker for the government, but it is also a key ingredient in many ancillary products like alcohol, drugs, chipboard, and paper. Pakistan is ranked seventh in sugar output globally and fifth in sugarcane cultivation. It accounts for about 2.5 percent of the agricultural sector's total value and 5 percent of the country's total cropped land. But despite its worldwide significance, Pakistan's sugarcane yield is lower than that of other countries. Various biotic stresses, such as insect pests, weeds, and diseases, which, when severe, significantly reduce crop output, are one of the many factors that contribute to this difficulty. Worldwide, sugarcane has been infested by up to 103 different insect pest species; 12 of these species have been documented in Pakistan alone. Among these, the infestation by the sugarcane stem borer (*Chilo infuscatellus*) stands out as the most notable. Estimates put the yield drop anywhere from 36 to 70 percent as a result of this. This pest's caterpillars munch through over 20% of a field's harvest every year. Caterpillar infestation of sugarcane stems lowers sucrose levels after harvest, increases stem breakage rates during adult plant stage, and increases seedling death rates, all of which lower the harvested crop's market value.

Chemical pesticide treatments have traditionally formed the backbone of conventional insect pest management in agriculture. Despite their usefulness in protecting crops from pests, these pesticides pose serious dangers to both humans and animals, and they have devastating effects on the environment. Classical breeding for insect-protected sugarcane cultivars is also fraught with difficulty because of the plant's lengthy and complex polyploid genome, which has over 120 chromosomes, and its small gene pool, which lacks resistance genes. To lessen the impact of such an infestation, it is crucial to seek out other solutions, such as the genetic engineering of insect-resistant

crops. This is why agricultural biotechnology has reached a new pinnacle with the advent of genetically modified crops that express the Cry toxins produced by the *Bacillus thuringiensis* bacterium, more commonly known as Bt. B. cereus is responsible for producing the crystal proteins that kill insects.

Bacillus thuringiensis is poisonous to *Lepidopteran* insects when they swallow it during the sporulation stage. In order to effectively manage stem borer insects, several agricultural species have had the Cry gene introgressed into them since the creation of Bt crops. Sugarcane, maize, cotton, soybeans, potatoes, and rice are among these crops. Traditional insecticide applications were greatly reduced after this technique was implemented, greatly improving pest control measures. A number of insect populations have developed resistance to Bt crops due to their extensive use. Field resistance to Bt endotoxins has been demonstrated in several pest species, including sugarcane stem borers *Diatraea saccharalis*, *Chilo partellus*, and *Busseola fusca* to Bt maize in South Africa. These kinds of developments have made the pursuit of alternate tactics an integral aspect of the ever-changing resistance.

A number of other genes have been identified, some of which show promise as prospective treatments for *Chilo infuscatellus*, joining the Cry gene in this regard. One such protein is Vip3, which is carried by Bacillus cereus and *Bacillus thuringiensis* and is responsible for protecting plants from insects. The Vip3 gene has also shown to be an excellent pest controller, and it has acquired commercial traction. In order to maximize its efficacy, the Vip3 gene is expressed in bacterial systems during both the sporulation and vegetative growth phases. Comparable to Cry proteins, Vip3A proteins cause midgut epithelial cells in insects to expand and osmotically lyse, which disrupts their function. Biochemical studies have shown that Vip3A is an 88.5 kDa protein with a variable C-terminal domain that dictates target selectivity and a highly conserved N-terminal region that aids in protein translocation across cell membranes. Protein Vip3's insecticidal action relies on its N-terminal region being intact; it has been found that mutations involving cysteine residues in this area can lead to the protein losing its insecticidal characteristics. Improving the sugarcane borer resistance of the local cultivar CPF-246 is the aim of this present study. Our transgenic sugarcane lines expressing the vegetative insecticidal protein Vip3A were resistant to the cane stem borer *C. infuscatellus* in both laboratory and field trials, as reported in this

paper. To overcome the difficulties of dealing with insect pests and to increase overall productivity and yield, this protein can be seen as a giant leap forward in creating insect-resistant sugarcane types. This cutting-edge technology has tremendous promise for the future of sustainable agriculture and for resolving the interconnected critical issues of food security in the face of total unmet global demand. Managing diseases and pests is crucial for achieving food security, especially for important cash crops like sugarcane. The use of resistant crop cultivars is one low-cost strategy for dealing with these problems. The sugarcane stem borer, scientifically known as *Chilo infuscatellus*, is among the most destructive pests in the industry. It cuts yields by 25–30% and reduces sugar recovery by roughly 15%. Conventional breeding for certain features, such insect resistance, is severely limited by the sugarcane genome's complicated genetic background. A potential alternative to chemical pesticides, based on these concerns, is the genetic alteration of crops to express transgenic proteins with insecticidal characteristics. More than 420 million hectares of land throughout the world have been used to cultivate Bt crops, which were made possible by introducing various genes (Cry1Ac, Cry2A, Cry1Ab, Cry1Aa3, Cry10Aa) from the bacterium Bacillus thuringiensis. In their initial research, the expression of the Cry1Ab gene in the sugarcane genome, marking the first instance of transgenic sugarcane developed for insect resistance. Insecticidal protein genes have been engineered into sugarcane in other research to make it more resistant to pest insects.

But the risk of insect resistance to the insecticidal proteins produced by Bt crops is a big worry for anyone planning to grow these crops. A major risk to the efficacy of Bt crops in the long run is this phenomena. The initial instances of resistance were documented as early as six years after the commercial debut of Bt-engineered crops, a result of the tremendous selective pressure on insect populations caused by their extensive use. There is an immediate need for effective techniques to control and mitigate resistance, as several studies have shown through thorough analysis that resistance has developed in numerous pest populations across continents. The use of two or more insecticidal poisons that target a single pest species—a technique known as gene pyramiding—has shown to be an effective strategy in combating pest resistance thus far. This strategy aims to prolong the effectiveness of transgenic crops by reducing the rate of resistance evolution in insect populations. So, the new vegetative insecticidal

protein Vip3A was the centre of attention in this investigation because of its possible use to sugarcane pest resistance. Notably, the Vip3A protein has a broad spectrum of activity against *Lepidopteran* species and does not share binding sites with any of the current Cry proteins. This makes it an intriguing candidate among these proteins.

There are a number of Bt cotton and Bt maize cultivars that have demonstrated promising results in managing insect populations. Some of these types express the Vip3A protein alone, while others combine it with Cry proteins. Vip3A protein levels in chloroplasts of transgenic plants were dramatically increased in a related work that fused the Vip3A gene to a chloroplast transit peptide-encoding region. Important pests' larvae, such as *Spodoptera frugiperda, Spodoptera exigua,* and *Helicoverpa zea*, were utterly eradicated by this treatment. A polyubiquitin promoter and two enhancers regulated the expression of the Vip3A gene in this investigation. Transgene expression in engineered plants was known to be greatly enhanced by these regulatory components, leading to improved resistance features. Polymerase chain reaction was used to confirm the transformation after GUS histochemical staining was used for initial screening of the converted sugarcane lines. Plants carrying the target transgene can be located using the PCR, a dependable and quick screening technique for transformants. After that, Southern blot analysis proved that sugarcane transgenic plants had successfully integrated the T-DNA region, confirming single copy insertion events. Stable integration of the T-DNA region, which typically occurs as low copy number insertions, has been demonstrated using biolistic transformation approaches, according to the biotechnological research on sugarcane. The known effect of transgenic expression on plant trait expression is well-documented. Our study's twelve transgenic sugarcane plants showed positive amplification results when targeted with gene-specific primers for the Vip3A gene. The same plants were further confirmed by the protein estimate, indicating that the transgene was well expressed. Transgenic sugarcane lines showed a range of Vip3A protein concentrations, from very low to very high. An 8.89 µg/mL concentration was noted in the S10 transgenic line, whereas an estimated 5.35 µg/mL was reported in the V5 transgenic lines, as the lowest value. The level of resistance developed against sugarcane borers is correlated with this variance in protein expression. The S10 transgenic line, which had the greatest Vip3A gene mRNA expression and a protein content of 8.65 µg/mL, resulted in a significant insect

fatality rate. Factors like copy number and the precise location of integration inside the host genome may explain why transgenic expression levels vary among events. Whether the foreign gene will rest in regions of transcriptionally active or inactive chromatin, and hence the expression level of the transgenic, is largely determined by the site of integration. Our findings support the idea that increasing the concentration of the Vip3A protein improves the effectiveness of pest management by increasing insect resistance, which in turn increases the levels of transgenic expression. This agrees with earlier research that found comparable associations between resistance and protein expression levels in transgenic plants. This proves that improving pest control requires optimizing transgenic expression. Previous research has established the impact of copy number variation on sugarcane transgenic lines' phenotypic traits, yield, and possible resistance to stem borer infestations, in addition to the results pertaining to Vip3A expression.

Screening, Stability, and Evaluation of Transgenic Traits

Next, the prospective transgenic plants were screened to initiate the molecular analyses, which included confirming stable integration and measuring the expression levels of the transformed transgenes. The lowest concentration for kanamycin screening was 50 mg/L, and plants tested positive for this compound were thought to be transgenic. Additionally, the expression level of the associated GUS gene was assessed using a GUS test. Actually, GUS activity was found in every section of the plant. Using a fluorescent microscope to examine GUS-positive sections of stem and leaves revealed that the GUS gene is expressed consistently throughout the plant. Our study demonstrated strong integration of several transgenes since the frequency of co-integration of connected genes reached 100%. It has been previously reported that fourteen separate transgenes can be successfully co-transformed into a single rice genome, lending credence to the idea that more genes can be incorporated into a single plant genome. A PCR analysis was performed on the hypothetical transgenic plants that were chosen using kanamycin and glyphosate selection media. Using gene-specific primers for PCR amplification, the tests showed that 15 plants screened from the lines in the V0 generation tested positive for CEMB-Cry1Ac + CEMB-Cry2A and CEMB-GTGene.

Southern DNA hybridization verified that the three transgenes were successfully integrated into the sugarcane genome. Through single and double digestions of the plasmids pCEMB-SGTG and pCEMB-SC12 with the restriction enzymes, the copy counts of the integrated transgenes were determined. Transgene copy numbers injected in agricultural plants using gene gun methods might vary from one to five copies, according to the literature. It was found in the V1 generation that Cry1Ac and Cry2A banding patterns were correlated with the intensity of hybridising bands, indicating that these transgenes might integrate as a single unit extremely frequently. There were no rearrangements seen in these transgenes. The successful incorporation of the transgene copies was verified by southern DNA hybridization.

According to most studies, the number of integrated gene copies can be anywhere from one to six, with most transgenics containing between one and two copies. We used enzyme-linked immunosorbent assays (ELISAs) and dipstick tests to measure the transgenic proteins' expression levels. All fifteen clones (V0) carried the CEMB-Bt and CEMB-GTGene genes, according to the dipstick-ELISA assay (immuno-strip assay), however their expression levels for these transgenes were significantly variable. The amount of copies of the transgene, its location on the genome, the presence or absence of heterozygosity at the sugarcane transgenic site, and both internal and external environmental variables all play a role in this variance. According to these research, non-homologous recombination is the mechanism by which foreign DNA in transgenic plants finds its way into chromosomal locations at random. The transprotein expressions for CEMB-Cry1Ac, CEMB-Cry2A, and CEMB-GTGene were 0.475 µg/g, 0.567 µg/g, and 0.486 µg/g, respectively, at the greatest time observed. In terms of expression levels of the three transgenes, lines CPF-246-(5L/5) and CPF-246-(6L/5) consistently exhibited the greatest levels, whilst lines CPF-246-(2L/8) and CPF-246-(5L/1) displayed the lowest. The field-grown V1 clones, which originated from the V0 generation and replicated to 150 copies, maintained their positive PCR amplification results when tested using gene-specific primers. We tested the integration stability and protein expression of CEMB-Cry1Ac, CEMB-Cry2A, and CEMB-GTGene in fifteen plants chosen at random from the V1 and V2 generations.

The genes with the greatest transprotein expression levels in the V1 generation were CEMB-Cry1Ac at 0.687 µg/g, CEMB-Cry2A at 0.611

µg/g, and CEMB-GTGene at 0.589 µg/g. When comparing CPF-246-(5L/5) and CPF-246-(6L/5), the expression patterns were comparable, but significantly lower in CPF-246-(2L/8) and CPF-246-(5L/1). Additional confirmation of the steady integration was provided by Southern blot analysis. These findings, which demonstrate consistent expression patterns and transgene persistence, are consistent with previous research on transgenic crops. After screening five V2 generation transgenic sugarcane lines, molecular studies verified that transprotein expression was stable across all five lines. All three transgenes were confirmed by PCR amplification, Southern blotting, dipstick assays, and ELISA in clones generated from the five lines. The expression profiles of all V2-generation clones were stable with respect to the transgene. Results from protein quantification using ELISA and glyphosate spray showed that glyphosate tolerance gene expression was consistently steady. Consistent with earlier studies, these results demonstrate that gene expression and inherited characteristics of integrated genes are stable over generations. Bioassays using the insect *Chilo infuscatellus*, which measures transprotein expressions of these integrated genes across generations V0, V1, and V2, corroborated the results. This work lays the groundwork for future research and applications in the field of transgenic sugarcane, demonstrating the success of the genetic alterations used to increase crop resilience and productivity. Using a leaf bioassay at four distinct time periods, the bioefficacy of the transgenes CEMB-Cry1Ac, CEMB-Cry2A, and CEMB-GTGene was assessed against *Chilo infuscatellus*, with a special emphasis on the second instar larvae of the shoot borer. Critically important is the quantitative level of toxins generated from these transgenes; these levels must reach sufficient levels during infestation to protect the crop adequately from attacks by the *Lepidopteran* shoot borers. During the first vegetative generation, V0, the quantities of toxins generated by CEMB-Cry1Ac and CEMB-Cry2A gradually reduced. Cane borer damage increases as a result of this. Specifically, for CEMB-Cry1Ac, the toxin levels decrease from 0.475 to 0.252 µg/g, and for CEMB-Cry2A, from 0.467 to 0.278 µg/g. Regardless of this decrease, compared to the controls, all transgenic lines showed a substantial increase in insect mortality, ranging from 60% to 100%.

In the following vegetative generation, V1, we tested the transgenic plants using leaf bioassays. There were a total of 15 lines, with 150 clones in each. When compared to non-transgenic plants, each transgenic line showed considerable improvement in leaf bio-toxicity

tests. Importantly, five of these lines—CEMB-Cry1Ac and CEMB-Cry2A—stably expressed their corresponding trans-proteins. When tested in the field, these plants exhibited no indication of insect damage. In contrast, the control plants suffered extensive damage. Transgenic sugarcane may be able to repel *Chilo infuscatellus* based on the partial browning of some plant leaves and the complete consumption of non-transgenic plants. Using ANOVA, LSD, and Dunnett's tests, the death percentages of *Chilo infuscatellus* larvae were compared to control and transgenic plants. When comparing the mortality rates of *Chilo infuscatellus* larvae among the transgenic sugarcane lines and the control plants, the results of the ANOVA and Dunnett's test showed that these differences were very significant at the 5% level of significance. The V2 generation had a death rate of 90% to 100%. According to LSD analysis, a considerably greater proportion of *Chilo infuscatellus* larvae mortality at 20 days (88% to be exact) was found in leaf samples collected from the CPF-246 (6L/5-4) transgenic line. However, transgenic line 5L/1-9 had the lowest death rate at 66%. This data, taken as a whole, confirmed the findings of earlier research by showing that there was a considerable difference in the percentages of leaf damage and insect death between the control and transgenic plants.

Integrating desired genes into a target plant's genome and passing them on to subsequent generations is crucial to the success of any transformation project. Over the course of three vegetative generations, researchers evaluated the transmission of genes with stab integration. This research provided compelling evidence that all three transgenes were amplified and integrated into the sugarcane genome in the V0 generation. Various methods, including PCR, Southern blot analysis, dipstick assays, and ELISA, were employed in this investigation. Additionally, V1 underwent southern blotting after having two copies of the altered genes inserted into the sugarcane genome. Analysis using enzyme-linked immunosorbent assay (ELISA) confirmed that the actively integrated genes were successfully translated into toxin-functional proteins. Generations V0, V1, and V2 of the *Chilo infuscatellus* fish were used to validate bioassays that relied on the presence of transproteins derived from the integrated genes. In addition to the bioassays, a glyphosate spray assay was performed to assess the transgenic sugarcane plants' tolerance to the glyphosate herbicide. The fact that weeds prevent the soil from holding onto vital nutrients makes them an obvious enemy of crop cultivation. Out of 300

transgenic sugarcane plants, 160 showed signs of glyphosate stress tolerance after 15 days of being sprayed with 3000 mL/ha of glyphosate. Glyphosate killed all of the weeds and caused necrotic signs in the other plants. Of the plants that survived the V1 generation's application of the same amount of glyphosate, 75% were able to do so, whereas 25% of the sensitive plants died in specific lines. This can be because of changes in plant responses and expression brought about by internal variables or by environmental stimuli. During the V2 generation, ten clone plants were evaluated from each of the five CPF-246 transgenic lines. These findings were previously published for related research. Tolerance to glyphosate treatment at 3000 mL/80 L/hectare was seen in all examined lines and their clone replicates during the V2 generation. The result is five glyphosate-tolerant lines that carry the CEMB glyphosate-tolerant gene, which was derived from the CPF-246 variety. Consistent with previous research, these findings demonstrate that the sugarcane CPF-246 genome contains the CEMB-Cry1Ac, CEMB-Cry2A, and CEMB-GTG genes. Stable expression and insecticidal activity were observed in transgenic plants from generations V0, V1, and V2. The amounts of toxin expression in the transgenic lines were verified by related molecular investigations across these generations.

This is a huge step forward in creating sugarcane cultivars that are resistant to cane borer and glyphosate by introducing the two Bt genes and tolerance to the plant's genetic makeup. Additional enhancement is possible with these transgenic lines by pyramiding other characteristics that have demonstrated high sugar output. Agricultural issues may be resolved in the end if a transgenic variety is created that expresses most of the desired traits. Concurrently, there is a growing movement worldwide towards sustainable practices that can help crops develop more natural defences against pests. Because of their typically inferior efficacy compared to traditional chemical controls, biological control methods that use naturally occurring bacteria, fungi, and viruses have garnered very little support. Agricultural yields have been increased through the use of insecticides, which have reduced losses caused by insect infestation. However, many countries have lost interest in using these potentially dangerous chemical sprays and pesticides, which has resulted in the de-registration of a few compounds.

Consequently, a remarkable strategy for increasing crop yields is the genetic engineering of crop plants to include resistant features. Plants can now be engineered to withstand insect borers, or crop genomes can be enhanced with several new resistance genes tailored to specific soil and weather circumstances. A cash crop and hot season crop, sugarcane may generate a lot of biomass. Pakistan needs agriculturally feasible circumstances, which are shared by the top five sugarcane producing countries in the world, if it wants to speed up its production. About a quarter of the world's sugar comes from sugarcane grown in Brazil, making it the biggest producer in the world. The remarkable versatility of sugarcane makes it an attractive raw material for many different industries. It can be transformed into biofuels, electricity, enzymes, drinks, alcohol, chipboard, paper confectionary, chemicals, plastics, synthetic materials, pesticides, and enzymes, among many other things. For these and other reasons, sugarcane is one of the most important agricultural commodities due to the significant impact it has on the economy. A new and promising way to improve the sustainability and productivity of sugarcane cultivation is through the improvement of transgenic varieties with increased pest resistance and herbicide tolerance. This will lead to increased agricultural resilience and contribute to global food security. Consequently, this area of agricultural biotechnology requires additional investment in R&D.

There is substantial evidence that transgenes with a medium copy number outperform those with a high or low copy number in terms of phenotypic traits and pest resistance. Because of the wide range of Vip3A gene integration and expression levels seen in these transgenic plants, it is clear that optimizing these parameters is crucial for maximizing the advantages of genetic improvement in sugarcane cultivation. Overarchingly, experimental results show that transgenic sugarcane lines with a single copy of the Vip3A gene in their genome are resistant to stem borers, suggesting a potential avenue for producing sugarcane varieties that are resistant to insects. The creation of transgenic sugarcane with resistance to insects may make use of these lines. Additionally, gene pyramiding techniques can be employed to mix them with pre-existing Bt toxins. Improving the resilience of sugarcane types toojí ao to changing insect and environmental stresses is a key objective of this accomplishment. Also, sugarcane, or *Saccharum officinarum*, is a Gramineae family perennial grass that is used to make gur, sugar, and ethanol, which is used both domestically and abroad. It is critical to increase sugarcane yield and

quality immediately because Pakistan is the world's fifteenth-largest sugar producer and the fifth-largest sugarcane producer overall. The desired increase in quality and target yield per acre has not been met, despite the increased demand for sugarcane. This is due to the fact that effective crop disease control and robust research initiatives are lacking. Due to the vegetative propagation of sugarcane types and the potential for illness during seed preparation and shipping, these plants have remained sensitive to a number of diseases.

A source of disease-free germplasm from Pakistan's well-adapted sugarcane cultivars is the goal of the current initiative. The goal of utilizing plant tissue culture techniques, specifically micropropagation and somatic embryogenesis, is to develop a more efficient method of sugarcane propagation that addresses the limitations of conventional breeding. Using these methods, premium sugarcane varieties can be mass-produced in a fraction of the time it would take using conventional methods, all without the risk of disease. Thus, the goal of the study is to isolate disease-free sugarcane germplasm from sixteen different varieties currently produced in Pakistan. In order to achieve this goal, we targeted genetic enhancement against the Sugarcane Mosaic Virus while optimizing a technique for the regeneration of plantlets using indirect somatic embryogenesis. To induce callus formation, we conducted tests with1.0,2.0,3.0, and 4.0 mg/L of the plant growth regulator 2,4-D. The time it took for callus formation, defined as the appearance of tiny nodular masses of cells at the cut edges of the explants, to occur was used to evaluate the effectiveness of these concentrations. Induction of calluses in sugarcane types was characterized by a wide range of textures, including woody, compact nodular, friable, and soft foamy, as well as a wide range of colors, from greenish yellow to pale yellow, golden yellow, and even whitish. We have seen that calluses that are tough, compact, and yellow with a whitish tone seem to be more embryogenic, however no comments were made regarding the optimal callus form. At both 3.0 and 4.0 mg/L, 2,4-D was well-received by all kinds, with 3.0 mg/L being the optimal dosage. The explants were effectively transformed into callus tissue when supplemented with 3.0 mg/L of 2,4-D, making it the most effective media supplementation for inducing callus formation. At 2.0 or 4.0 mg/L, for example, it seems to have less of an impact, since the conversion is slower and less thorough. Embryogenic induction happens in some kinds under 3.0 mg/L supplementation, and our experiments showed that explants grown for six to nine weeks entirely

changed into callus tissue. Using these tissue culture procedures to increase the disease-resistant capacity and quality excellence of the F1-sugarcane germplasm has shown promising outcomes. Now that the sugarcane industry in Pakistan has entered a critical period where raising production and managing diseases are of the utmost importance. Many researchers have found that 2,4-D is the most effective chemical for inducing calluses in various plant species. Furthermore, numerous research have shown that it is quite effective.

Further Readings

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CHAPTER 5: **PGPR**

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Role of Plant Growth-Promoting *Rhizobacteria* (PGPR) in Enhancing Soil Nutrient Availability and Crop Yield

Plant growth-promoting *Rhizobacteria* are a heterogeneous group of bacteria inhabiting the soil, helping in improving the growth and development of crop plants through various mechanisms. These microbes create microbiological niches in the rhizosphere, defined as that part of the soil which is in direct contact with plant roots. In this ecological perspective, these PGPR significantly enhance water uptake, essential ions, nutrients, and soluble salts from the soil and contribute to the plants' vegetative and reproductive potential, which eventually results in increased yields. Such interaction between plant roots and PGPR is characterized by the release of organic compounds from the roots, which includes organic acids, sugars, vitamins, phytosiderophores, amino acids, nucleosides, mucilage, and various plant metabolites. Exudates act as a chemotactic signal that attracts PGPR in the rhizosphere and enhances microbial colonization, progressing towards mutualistic relationships. By this interaction, PGPR improves nutritional bioavailability in soil through mechanisms mainly involving phosphate solubilization, production of phytohormones similar to IAA, and nitrogen fixation. Such mechanisms are necessary to improve the nutritional conditions of plants, especially in unproductive soils. One of the important functions PGPR can perform is phosphate solubilization, converting insoluble phosphorus sources into soluble forms like tricalcium phosphate, which plants can easily take up. In the present study, different bacterial strains were subjected to their efficiency regarding tricalcium phosphate solubilization, and it was observed that these PGPR strains produced big halo zones on NBRIP media. This finding is in agreement with earlier reports on other crops and indicates that phosphorus solubilization is a general trait of PGPR.

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Moreover, many strains of PGPR exhibited the production of IAA, which is one of the most common phytohormones produced by a bacterium that helps in plant growth. This secretion is considered very important for the development of roots and overall vigor of plants. In the current study, out of 11 phosphate-solubilizing bacteria isolates, 9 produced IAA through pathways independent of tryptophan. Its maximum production was after 48 hours of growth and decreased afterward at 72 hours. That is important because at least with some bacteria it points to a probability of differential impact on plant growth over time. In fact, though some literature reports that the strains like *Klebsiella pneumoniae* are effective in producing IAA, IAA production of isolates showed a decline after 48 hours, which suggests that various strains may employ different metabolic pathways for synthesizing IAA. The findings of the present study further underscore the importance of the screening of native isolates of PGPR for their utility in bringing significant benefits within agricultural scenarios. It involved ombined application of the two PGPR strains, namely CEMB-15 and CEMB-22, enhanced rice crops' growth and productivity particularly. It demonstrates the synergistic action from multi-strain PGPRs in developing bio-fertilizer technologies that could either substitute for or complement the use of chemical fertilizers for improved agricultural sustainability.

Expanding the Role of PGPR

Besides this, nutritional availability with increased growth is not the only form of stresses PGPR has been able to confer on crop plants. They may be helpful to the plant in coping with abiotic stresses such as heavy metal contamination and other environmental pollutants. Some PGPR can also be utilized as phytoremediation agents for enhancing the bioremediation potential of contaminated soils by enhancing the plant's capacity to sequester heavy metals and improve soil quality. The correlational studies of the present research show that selection of rice genotypes might be done based on significant genotypic correlations among different traits, which may explain the usefulness in the development of higher-yielding varieties. In the investigation of correlation phenotypic and environmental, PGPR strains CEMB15, CEMB22, or a combination of both strains could be favorable for rice productivity enhancement.

It is also further suggested that selecting higher-yielding rice genotypes based on genetically associated traits could be a valuable strategy in crop improvement, further supported by direct and indirect positive effects observed in the study. Principal component analysis gives the contribution of individual traits to overall crop yield that shows some selected traits exert more influence on rice productivity. Organic compounds may, therefore, accumulate in the plant system with the inclusion of PGPR and enhance its yields. We have determined the potentials for combined PGPR applications in the optimum growth and productivity of rice crops. Presently, these findings are viewed in the broad context of impact assessment arising from the application of PGPR in agrarian systems. More research will be conducted to see the effect PGPR, especially CEMB-15 and CEMB-22, can have on other major crops like maize, wheat, sugarcane, and cotton. In this way, the adaptability and versatility of these PGPR for an extended range of crop species will be understood under realistic conditions, thereby even realizing their general value in agricultural applications. With PGPR being injudiciously released into the agroecosystem, there is a need to look into certain possible effects that would be considered negative impacts on the indigenous microbiota. Ecological balance in soil can be fragile, and the addition of alien bacteria needs to be carefully managed to avoid compromising established microbial societies. Long-term effects of application should be investigated regarding soil health, microbial diversity, and plant relationships for its sustainability. This emphasizes the incorporation of PGPR into crop management strategies as a promising approach toward improving agricultural productivity while minimizing the dependency on chemical fertilizers. Farmers, by exploiting the beneficial properties of such bacteria, can improve soil health and increase crop yields to promote sustainable agriculture methodologies. There is a need to look in depth at other aspects, such as mechanistic studies, accessible practical applications across crops, and the likely ecological implications of PGPR in holistic views of sustainable agricultural practices.

Comprehensive Characterization of Phosphate-Solubilizing Bacteria (PSB)

The current study applies a polyphasic approach-morphological, biochemical, and molecular methods-for identification and characterization of the PSB isolates. Consequently, the morphological

method consists of colony and cell morphologies in addition to Gram staining. Then again, the biochemical test encompasses oxidase, catalase activity test, and quantization test by using QTS-24 system. Furthermore, the molecular method includes 16S rRNA gene sequencing for an accurate species identification to the obtained PSB strains. Notably, all the isolates did show phosphate solubilization after three days of inoculation. This could be due to the fact that favorable conditions for PSB growth lead to acidification of the growth media, as reported in the literature, with a linear increase in supernatant soluble phosphorus related to the bacterium growth. The isolated PSB strains were also able to produce indole-3-acetic acid, a plant growth regulator that is greatly responsible for plant growth and development. Previous works have shown that the PSB strains isolated in diverse environments including rhizosphere of aerobic rice showed a key impact on the enhancement of plant growth due to their ability to produce IAA. In addition, the dual culture on PDA plates showed that PSB strains were antagonistic to the pathogenic fungus Fusarium. This result agrees with those of earlier works where it was recorded that PSB isolates, especially members of the genus Pseudomonas, represented the maximum percentage of antagonistic activities against various plant pathogens. The correlation analysis among the selected PSB isolates revealed a strong and significant relationship among some isolates, including R-1, R-2, R-3, SC-01, SC-03, SC-05, SC-07, SC-19, SC-20, SC-17, and SC-22 in respect to phosphate solubilization percentage. It is worthwhile to say that combinations of different isolates may further enhance phosphate solubilization in plants, hence potentially improving crop yield and potential. The selection of efficient PSB isolates could significantly enhance the plants' ability to utilize and absorb more amounts of phosphates, which would in turn lead to higher agricultural productivity with time.

It also showed that the phosphate-solubilizing *Rhizobacteria*l isolates, including R-1, R-3, SC-01, SC-03, SC-07, SC-09, SC-13, SC-19, SC-17, SC-20, and SC-22 had a good correlation with each other, presenting antifungal microbic activity against Fusarium for growth-free inhibition zones. This can be interpreted as a high degree of correlation, suggesting that the combined application may be more efficient in antifungal activity than the application of individual components. The synergistic action among different isolates enables the formulation of a more effective biocontrol strategy against pathogenic fungi in agricultural environments. Salinity is thought to be one of the major

threats to agriculture and it has been affecting many regions in the world for more than 3000 years. With the rising population in the world, the demand for food from society also increases, which means that more agricultural land is being used and higher production per unit area. Approximately 20% of the irrigated lands are salt-affected, and the total area estimated to be salt-affected by soils is about 830 million hectares. Salinity affects precipitous deterioration of agricultural lands but leads to adverse effects on economic advancement and nutritional standards. There is an active engagement of plant scientists in developing salt tolerance in varieties using various genetic approaches. However, because of the poor understanding of the biochemical, molecular, and physiological mechanisms involved in the interplay of salt stress, its effective development of salt tolerance is delayed. Many of the stress factors exert their adverse effects on growth by causing hormonal imbalance, making plants more prone to diseases, and resulting in nutritional disorders in them. From this very urge to enhance salinity tolerance, over the years, the Ventura of biological agents has come into prominence. These beneficial microorganisms, popularly known as PGPR, help in maintaining hormonal and nutritional balances, solubilization of nutrients, production of plant growth regulators, and inducing resistance mechanisms in plants. In fact, PGPR has been considered an effective bioremediation agent for treating saline soils through a variety of direct and indirect mechanisms. These are peculiarly beneficial in the rhizosphere, the area around plant roots where intense biochemical interactions under the action of root exudates take place.

Phosphate-Solubilizing Bacteria (PSB)

Modern agriculture has been increasingly dependent on chemical fertilizers as the most trusted form of essential plant nutrients due to increasing demand with global requirements of food. However, such heavy reliance on synthetic fertilizers has sparked several concerns about sustainability among environmentalists and agricultural scientists. Continuous application and excessive use of chemical fertilizers have long been found associated with adverse effects on soil health, environmental pollution, and a reduction in the diversity and activity of the beneficial soil microorganisms. In the backdrop of these menaces, all too increasing awareness for incorporating organic amendments and bio-fertilizers in agriculture is to preserve soil health and improve plant productivity.

Of all the macronutrients required for plant growth, phosphorus is one of the most important limiting factors in productivity, with nitrogen as its precursor. Phosphorus performs an important function in energy transportation within a plant, in photosynthesis, and in the synthesis of nucleic acids and phospholipids. The general availability of phosphorus to soils is very limited because it tends to be rapidly fixed into elements where it is not available to plants. It was seen that as much as 75% in some soils of the applied phosphate fertilizers can re-precipitate and result in a large loss of potently available phosphorus to plants. Unlike nitrogen, which is abundant in the atmosphere, phosphorus does not exist in a form that can be readily exploited by plants, indicating the need for novel strategies that enhance its bioavailability.

More recently, PSB has emerged as a very important and vital group of microorganisms that enhance phosphorus availability in agricultural systems. These valuable microorganisms have the unique ability to convert insoluble phosphate compounds into soluble forms available for ready absorption by plant roots. Their contribution to the dissolution of different forms of phosphorus, including tricalcium phosphate, underlines their role for the betterment of phosphorus deficiencies in soils. Historically, naturally occurring rhizospheric phosphate-solubilizing microorganisms have been recognized for over a century to stand out with their long-time potential to improve the growth of plants together with soil fertility. Phosphorus, especially, plays an important role in growing specific crops such as sugarcane, where the yield is not the only factor but also the quality of the sugarcane juice. Application of phosphorus fertilizers has become an integral part of the package of practices related to sugarcane cultivation for maximum productivity. There is now an increasing interest by researchers in the use of purified strains of PSB for assessing their efficiency in phosphate solubilization and plant growth promotion, especially in genetically modified varieties of sugarcane that have developed resistance against certain diseases such as the Sugarcane Mosaic Virus disease. *Pseudomonas, Bacillus, Rhizobium, Micrococcus, Flavobacterium, Burkholderia, Achromobacter, Erwinia,* and *Agrobacterium* are some of the bacterial genera reported as efficient phosphate solubilizers. Besides acting as a biofertilizer, these microorganisms contribute to enhancing agricultural productivity through various mechanisms. Pseudomonas species have especially been reported to possess multiple biofertilizing properties. Thus,

besides their capability of improving soil nutrient status, they possess properties related to the production of plant growth regulators and suppression of soil-borne pathogens. Besides that, the species Bacillus are named as auxin producers, which further contribute to their efficiency in plant growth promotion.

Exploring the Role of Phosphate-Solubilizing Bacteria (PSB) and Plant Growth-Promoting *Rhizobacteria* (PGPR) in Sustainable Agriculture

In this light, the application of biocontrol agents or organisms, including beneficial bacteria, may turn out to be a promising and eco-friendly approach for agriculture other than inputs based on chemicals. Rhizosphere-associated organisms have been reported to produce a wide array of growth-promoting substances that influence morphology and plant health substantially. This relationship thus highlights the potential to exploit natural microbial communities as an approach for enhancing agricultural productivity. Plant growth-promoting *Rhizobacteria* are diverse groups of bacteria that colonize the rhizosphere and enhance plant growth through various mechanisms, including the production of phytohormones, nutrient solubilization, such as phosphorus, and inducing systemic resistance against pathogens. Such an association with different PGPR isolates presents an opportunity for selection and combination of strains that synergistically enhance plant growth and yield in an environment. In view of the growing international concern for food security and environmental quality, the use of PGPR in sustainable agriculture is of prime importance. Various formulations were developed, and many of them showed increased growth with improved seed emergence, which enhanced crop yield. Certain of these products are already commercialized.

Establishing the relationship among such isolates in order to explore using them together for improvement of phosphorus availability to crops and overall plant health is very important. Phosphorus is one of the most important macronutrients for plant development, while most of the applied phosphorus through chemical fertilizers turns into insoluble Ca or Mg salts in soils, which are not available to plants. These organisms, along with PSB, are important in the conversion of insoluble phosphorus to soluble forms, making it more available to

plant roots. PSB plays an important role in agricultural soils, since these microorganisms directly or indirectly influence the soil health through their beneficial activities. Thereby, the rhizospheric microbes are known to play a crucial role in mediating fundamental processes in the soil, such as decomposition of organic matter, mineralization of nutrients, and nitrogen fixation.

Characterization of Phosphate-Solubilizing Bacteria (PSB) for Enhanced Plant Nutrient Availability

The mechanism of mineral phosphate solubilization by PSB generally involves the production of low molecular weight organic acids. Organic acids containing hydroxyl and carboxyl functional groups interact with cations bound to phosphate, thus facilitating the conversion of phosphate into soluble forms. This process of phosphate solubilization is very complicated and is influenced by various nutritional and physiological parameters, besides cultivation methods. Growth of PSB in media having calcium phosphate as the sole phosphorus source forms a basis to study their solubilizing activity in terms of a clear halo or zone around the bacterial colonies on solid medium.

However, halo formation alone is not good enough to rely on for the detection of potential PSB, but rather it needs the development of complementary methods for full assessment. The optimal condition for isolation and purification of PSB from the rhizosphere soil of various plant species is presented here. The protocol consisted of the isolation and identification of PSB through physiological and biochemical tests, followed by testing the bacterial plant growth-promoting effects in greenhouse conditions. It was envisaged that one of the most effective ways of improving nutrient availability within plants would involve the use of PSBs-particularly phosphorus, which is imperative in the metabolic activities of plants. Isolation and characterization of phosphate-solubilizing bacteria from the rhizospheres of different plants provided important information about their functional role in nutrient availability. The capability of PSBs to solubilize tricalcium phosphate, as reflected by clear halos on Pickovskaya agar plates, points toward their efficiency in converting insoluble phosphorus into bioavailable ones. This trait is of prime importance from an agricultural point of view because most soils contain inadequate levels of readily available phosphorus for crop productivity.

Among the tested twelve isolates, a maximum SI of 2.23 was identified by BR2 isolate, which showed its better ability in phosphorus solubilization. Along with confirming BR2 as a potent PSB strain, it also proved the presence of variability in P solubilizing abilities among different bacterial varieties. The occurrence of such variability insists the selection of specific PSB strains as a potential approach for increasing soil fertility and crop yields under various agricultural conditions. The morphological study of PSB isolates revealed that they maintained rounded,-raised colonies with smooth surfaces, typical for the beneficial bacteria of the soil. Further, one striking feature was that a certain group of these isolates was found to be Gram-negative. Many of the well-documented species capable of phosphate solubilization belong to this category of Gram-negative nature. Distinct color variations in these isolates might suggest variable metabolic capabilities or ecological adaptations, therefore opening new ways of studying their roles in the rhizosphere. Various biochemical tests, like catalase and oxidase assays, provide endpoints of physiological characteristics related to the PSB isolates. The positive catalase activities found in some of the PSB isolates suggest that these isolates have the potential to reduce oxidative stress, thereby enhancing their survival and functionality under a wide range of soil conditions. This is an important factor affecting resilience for effective plant-microbe interactions, especially under conditions that are not good for bacterial functioning. Advanced techniques of identification, widely included in work practice, such as QTS-24 Miniaturized Quantification System, have provided a new and exact way of classification for bacterial dominant representatives in the PSB community. The species identified, such as *Pseudomonas fluorescens, Klebsiella pneumoniae,* and *Proteus vulgaris* express functional importance regarding plant growth promotion and nutrient uptake.

Enhancing Crop Growth and Nutrient Uptake in Sugarcane

In this work, the authors set the basis for further studies concerning the ecological functions and interactions these PSB may enter in the rhizosphere using modern methods of microbial identification. The greenhouse experiments, which were conducted to evaluate the performance of selected PSB isolates together with the genetically modified sugarcane varieties (CAMB I and CAMB II), hold promise in

bringing several efficacies on the growth and development of plants. The excellent increase in plant height, up to 1103%, associated with BR2 isolate in CAMB II, is illustrative of the potential of this isolate to substantially improve key growth metrics. Besides, the differential response of the two sugarcane varieties suggests that the success of PSB inoculation may be genetically determined so that future applications could become cultivar-specific. Additionally, besides an increase in height of the plants, PSB inoculation collectively led to a higher number of leaves per plant with increased shoot and root lengths, which further established its effect on general plant development exerted by useful microbes. The relationship between activities of PSB and improved nutrient uptakes is probably contributory to improved growth parameters. This boosts photosynthetic efficiency due to increased leaf area and biomass, an aspect that is very important for maximizing crop productivity; hence, focusing on practical implications of using PSB as biofertilizers at farm levels. Moreover, the higher density of root hairs in the PSB-inoculated plants, as compared to controls, evidences that these beneficial microorganisms positively influence root architecture. A more elaborate and developed root system enhances a plant's capacity for water and nutrient absorption, hence promoting overall growth performance. Such roots morphological changes are very important in ensuring optimum plant performance, especially under nutrient-poor soils where efficient nutrient acquisition is a prerequisite for maintaining crop productivity.

The increased shoot and root dry weights of the inoculated plants support this involvement of PSB by biomass accumulation. The most striking increases, especially in combinations of CAMB I/BR2 and CAMB II/PR2, speak to the efficacy of these respective isolates in the promotion of better growth development in plants. This increase in biomass is one of the major parameters controlling overall yield and productivity in crop plants, thus implying that the application of PSB in agricultural field practices would be a sustainable step toward better food production. Phosphorus is assumed to be one of the basic macronutrients requiring plant growth and development. The main route of phosphorus uptake by plants is via the solution phase-where the concentration is very low on account of low solubility and fixing in soils. At any one time, only a very small proportion of potentially available phosphorus is available to the plant. $H_2PO_4^-$ and HPO_4^{2-} are taken up by plants through the roots as the main forms, which varies

with the changes of $H_2PO_4^-$ soil's pH. Some bacteria, known as PSB, have the capability to solubilize the insoluble forms of phosphorus compounds and make them available to plant roots, which is very efficient in increasing their availability to different soils. The populations of PSB are ubiquitous in nature and fluctuate according to the type of soil in which they are found. They usually form up to 1-50% of the total microbial population present in the soil and are more concentrated in the rhizosphere, where they usually show a higher metabolic activity compared to those isolated from other environments. The first records of the occurrence of natural rhizospheric PSB indicated an important role played by these isolates in plant growth and nutrient uptake. The most efficient phosphate solubilizers were found to belong to the genera *Pseudomonas, Bacillus, Rhizobium,* and *Enterobacter*. The major mechanisms through which PSB enhance phosphorus availability mainly include the production of organic In the process, there is acid production that acidifies the surrounding medium. Acidification and the solubilizing action of PSB transform insoluble phosphorus into easily absorbable forms by plant roots. Pathogenic microbes are causing immense yield losses and pose great threats to global crop yield in terms of attainment of food security and stability. In modern agriculture, agricultural activities were intensified to meet the increased demand for food; this is followed by mass application of agro-chemicals to safeguard crops against these devastating pathogens. Unfortunately, the rampant use of chemical pesticides and inorganic fertilizers has been linked with serious detriments to non-target organisms and ecosystems, hence the urgent requirement for practising sustainable agriculture.

Enhancing Crop Growth, Alleviating Salinity Stress, and Supporting Sustainable Agriculture

PGPRs inoculating phytohormones are efficient at enhancing plant growth and yield, especially phosphorus, nitrogen, and potassium nutrient acquisition in soil due to solubilization. In addition, PGPR can also act as a biocontrol agent by the production of siderophores, antifungal metabolites, and under competition for specific niches on the plant roots. Various responses of plants to salt stress have been recorded at the cellular, tissue, and whole-plant level. PGPR treatment cansın markedly alleviate the adverse effects of salinity on plant growth and, as such, are a valuable ally in striving for sustainable

agriculture. There are many other ways and strategies that have been developed and still are being researched and developed through which PGPRs help plants mitigate any form of loss resulting from abiotic stresses. The continuous studies on the synergistic implications of PGPR along or in combination with other beneficial microorganisms show great potential for enhanced crops resilient to increasing soil salinity and other abiotic stresses. In this way, through an extensive study for isolating and characterizing efficient PGPR and PSB, this agricultural sector can enter into more sustainable and eco-friendly areas, which will advance the interests of crop yield together with ensuring soil health and ecosystem integrity. A hyperosmotic shock reduces the chemical activity of water, inducing a loss in turgor pressure in plant cells. This triggers several stress responses, those being related to nutritional imbalances, hypoxia, and hyperosmotic stress. From all abiotic stresses, salinity represents one of the most common environmental stressors that among all others impair plant growth and development. The injurious action of salinity may be considered to act in two stages: an immediate osmotic effect of preventing the intake of water by the plant, and after some days, a progressive accumulation of salt toxicity in the tissues. This latter phase is survived only by those plant species that have developed mechanisms for tolerance of salt stress by preventing the accumulation of toxic salts in their leaves.

The effect of saline environments on plants is manifested as a reduction in major morphological and physiological attributes, which reflects as a reduction in overall growth. The losses in turgor due to the quick osmotic effect reduce the efficiency of water uptake, thus restricting growth. Such growth reduction can be quantified by determining the yield of the salt-stressed plants. Vegetative growth has been reported to be more susceptible to salt stress than reproductive growth, indicating partial tolerance at various growth stages.

Enhancements in Morphological and Biochemical Parameters

The present investigation evaluated the effects of four specific *Rhizobacteria*l strains on morphological and biochemical parameters of tomato plants subjected to salt stress. Salt-treated but untreated

controls showed a significant decrease in all the morphological parameters such as shoot length, root length, shoot mass, total plant weight, root weight, leaf surface area, and number of leaves. The first event of salt stress in plant cells is dehydration and shrinkage. After several hours, these cells may regain some turgor pressure; however, the succeeding events of cell elongation and division become compromised. Compromised cell elongation and cell division lead to retarded growth of leaves and roots. The effect is more pronounced on leaf emergence, which is slower, and the leaves larger with shoot development showing a later sign of salt damage. A high concentration of accumulated salts in the soil inhibits root water-extraction capacity. These majorly differentiate the growth of salt-stressed plants from the non-stressed ones. In this paper, plants treated with PGPR displayed healthier green leaves, while in those cases where plants were subjected to salt stress, leaves became yellowish, which is characteristic of nutrient deficiency or any stress-induced chlorosis.

These findings are in agreement with the available literature showing that salinity has the potential to reduce tomato fruit size by impairing water transport mechanisms. PGPR is a heterogenous group of bacteria which colonizes the rhizosphere and exerts beneficial effects on plant growth and development in various direct and indirect ways. These bacteria enhance nutrient use, solubilize key nutrients such as phosphorus, nitrogen and potassium, and also produce plant growth regulators. Besides that, PGPR can also act as biocontrol agents by protecting plants against phytopathogens through the production of siderophores, which are iron chelating agents making this element available both to the bacteria and to the plant.

Physiological and Biochemical Responses of Tomato Plants to Salt Stress

Salt stress triggers a series of complex physiological and biochemical responses in multifarious levels from cells to tissues to the whole plant. It is the biochemical pathways involved in improving the water retention capacity of the plant that plays a very important role in deciding salt tolerance. However, the biochemical toxicity due to salt stress impairs nutrient assimilation and uptake causing further

restriction to growth. In the present study, irrigation of saline water to tomato plants caused significant reduction in general growth as manifested by reductions in fresh weight, dry weight, leaf number, and other morphological attributes. The plants treated with PGPR showed higher amount of chlorophyll, carotenoids, and anthocyanins compared to non-treated salt-stressed plants. The increase in chlorophyll is mainly because of the fact that iron is an essential element of the biosynthesis of chlorophyll; it is effectively chelated and made bioavailable by PGPR through the production of low-molecular-mass iron chelators called siderophores.

This is the mechanism employed by Gram-negative and Gram-positive *Rhizobacteria* to reduce Fe3+ in the Fe3+-siderophore complex; this maintains iron in a bioavailable form for plant use, thus promoting photosynthetic activity and overall plant growth. The above role of PGPR in the modulation of nutrient availability is further confirmed by their ability to increase the solubilization and assimilation of those essential nutrients. The direct interaction of PGPR with plants has been related to the direct influence exerted by PGPR on plant growth and development. Indirectly, these bacteria can decrease, in some way, the impact of a great variety of pathogens, and there is evidence that PGPR can induce a systemic tolerance against salt stress. Indeed, previous investigations have established that individuals belonging to various PGPR strains, among them Pseudomonas species, enhance the dry weight of plants, nodule formation, chlorophyll content, and overall seed yield in different crops. Corroborating these observations, experiments conducted under salt stress conditions with Psychrobacter species have also reported improved plant growth and biomass accumulation in several plant species tested.

The Role of Bacillus Species in Growth Promotion and Stress Resilience

A number of other works that utilized Bacillus species as PGPR have also reported significant growth parameters, nodulation, and heavy metal uptake in crops. The significant positive correlations among morphological and biochemical traits in the tomato plants treated with PGPR in the present study suggest that the use of such beneficial bacterial strains may play a key role in the development of resilience in tomato plants against salinity stress. The present study underpins the

urgent need for the exploitation of PGPR to develop strategies aiming at mitigating the injurious effect of salinity on crop production. Plants respond to salt stress through various cellular, biochemical, and whole-plant mechanisms. Salt tolerance determination involves specific biochemical pathways that enhance the water-retainment capacity of plants. Conversely, biochemical toxicity as regards salt stress causes an inhibition of nutrient assimilation and its uptake, resulting in widespread growth limitation. It is clear from the above results that irrigation with saline water had significantly affected tomato plants' general growth, as reflected by a reduction in all the studied physical traits, including fresh weight, dry weight, and number of leaves. Chlorophyll, carotenoid, and anthocyanin contents were also increased in PGPR-treated salt-stressed plants compared with untreated ones.

These bacteria in the soil acquire their iron by producing low-molecular-weight iron chelators that are known as siderophores. In Gram-negative as well as in Gram-positive *Rhizobacteria*, the Fe3+ within the Fe3+-siderophore complex is reduced. Thereby, the iron gets available to both the plant and the bacteria. Iron is an essential component for chlorophyll formation. Iron availability through PGPR enhances the photosynthetic activity and hence overall growth of the plants. This is further supported by the fact that PGPR enhance the solubilization and uptake of essential nutrients. PGPR modulates growth and development of plants through enhanced nutrient availability. In fact, PGPR indirectly counteracts the effects of destructive organisms. PGPR have been found provoking induced systemic tolerance to salt, thus directly functioning as growth promoters and protectors. Besides, PGPR application has been associated with significant increase in growth among different crops.

Leveraging PGPR for Enhanced Growth, Yield, and Stress Tolerance in Crops

One of the most striking works demonstrated that there was enhanced dry weight, chlorophyll content, and overall yield by the application of Pseudomonas species to Greengram. In other works with Psychrobacter species for *Ricinus communis* and *Helianthus annuus*, there was stimulated growth with enhanced biomass under salt stress conditions. Moreover, strains of Bacillus used as PGPR for chickpea have increased growth and yields by reducing accumulation of heavy

metals within different plant tissues. In the present study, the correlations among different morphological and biochemical attributes of PGPR-treated tomato plants bring out vividly the vital role these beneficial bacteria can play in improving plant growth and tolerance against adverse conditions of salt stress. These findings point to the possibility of using PGPR as a strategy for enhancing agricultural productivity, especially in salinity-prone environments. These complex interactions between PGPR and host plants build not only plant resilience but also make a case for continued exploration of these microbial agents in order to improve agricultural practices and reduce challenges provided by abiotic environmental stressors such as salinity. Elucidation of the complex mechanisms involved in modulation of plant growth by PGPR will help explore agricultural strategies aimed at assuring food security in an increasingly saline world. While researchers continue to study the diverse roles of PGPR, there is little doubt that integration of these beneficial bacteria into agricultural practice will contribute to the development of more sustainable farming systems able to tolerate the vagaries of environmental stresses. Plant growth promotion, improvement in nutrient uptake, enhancement of resistance against salt stress by applying PGPR will be important in future scenarios over the change in agricultural practice to support global food production against the increasing trend of soil salinity. Bell pepper or, popularly known in local dialect as "Sagia Mirch", sweet pepper, bullnose pepper, and "Shimla Mirch", is one of the most highly marketed vegetables in South Asia. Due to its bright colors and unlike any other flavor profile, bell pepper landscaping is walloping with essential nutrients. Besides its food value, the pepper is valued high for its pungency, which is in use in the manufacture of balms; much more for its carotenoid pigments used as color additives in poultry, food, and prawn feed industries.

However, there exist different agronomic methods to improve the yield of sweet pepper. Application of plant growth-promoting *Rhizobacteria* is a promising approach. Plant growth-promoting *Rhizobacteria* are beneficial, soil-based bacteria that colonize the rhizosphere-the soil environment immediately surrounding a root and influenced by root secretions. Application of PGPR can facilitate resource acquisition or directly inhibit the growth of plant pathogens, particularly fungi.

PGPR impact on yield

The rhizosphere environment is dynamic, with roots exuding organic compounds, such as organic acids, sugars, vitamins, phytosiderophores, amino acids, nucleosides, and mucilage. Such exudates attract beneficial microorganisms and build up a complex microbial ecosystem. It is these interactions that exist between the PGPR and plant roots which play an essential role in improving nutrient availability. For instance, some PGPR strains have the capability to solubilize phosphorus, fix nitrogen, and produce phytosiderophores that increase the nutritional status in the plants. These PGPR also improve root activities of crop plants through various mechanisms, including enzymatic activities and symbiotic associations with mycorrhizae or rhizobia, thus leading to a healthier root system with better nutrient uptake and overall plant growth.

A recent study, therefore, which is centered on the effects of PGPR on the yield of bell pepper, showed that the PGPR significantly increased the yield per treatment and per acre for an application rate of 6 liters per acre. Moreover, selection for an increment in fruit yield was a promise in genetic gains studies of synthetic varieties. High estimates of heritability indicated that hybrid development could be a promising avenue for improvement of yield per plant. The genotypic correlations indicated that selection for increased yields per acre may be done based on the yield per treatment evaluation. The positive values of the phenotypic correlations did establish the potential importance of PGPR with regard to improving the yield of bell pepper. Anomalously, the use of PGPR will not only advance the performance of bell pepper but also could be used in improving the yield of any other crop species. Results from numerous studies have underlined the potential of PGPR as an essential ingredient for sustainable agriculture, enabling the use of a biological method to improve crop yields. The successful use of PGPRs may open up more research activities targeting the assessment of their effects on other staple crops such as maize, wheat, rice, and barley. Ultimately, it may lead to wider application of PGPR in diverse agriculture systems with the contribution toward better food security achieved by sustainable farming.

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About this Book

This book takes an in-depth look at the economic importance, challenges, and biotechnological innovations impacting major agricultural crops like cotton, maize, potatoes, and sugarcane. Spanning five comprehensive chapters, the text explores how genetic engineering, pest management strategies, and sustainable agricultural practices can enhance crop productivity, quality, and resistance.

The opening chapter discusses the economic significance of cotton production and the critical role of transgenic approaches in combating insect pests and weeds. It examines the benefits of Bt and glyphosate-resistant genes, as well as advancements in RNAi and viral resistance for tackling cotton leaf curl disease. The maize chapter subsequently covers pest and weed management, development of insect-resistant transgenics, and the potential of chitinase for sustainable pest control.

A full section is devoted to the role of plant growth-promoting rhizobacteria (PGPR) in improving soil nutrient availability and crop yields across various agricultural systems. The text characterizes phosphate-solubilizing bacteria and explores their applications for enhancing sugarcane growth, alleviating salinity stress, and supporting sustainable practices.

The potato chapter explores RNA silencing strategies and genetic engineering for achieving viral immunity, while also addressing mechanisms and innovations in disease resistance more broadly. Finally, the sugarcane segment explores genetic engineering for disease and virus resistance, herbicide tolerance, and optimizing sucrose production and sugar isomerization through microbial applications.

Featuring the latest research and case studies, this comprehensive volume equips readers with a thorough understanding of how biotechnology and sustainable agriculture can address the mounting challenges facing major crop production worldwide.

About the Editor

Prof. Dr. Idrees Ahmad Nasir is a distinguished scientist and researcher with over 25 years of experience in advancing agricultural research and operational optimization across national and international fields. Known for his strategic insight and dedication to research-driven innovation, Dr. Nasir has a unique ability to translate scientific vision into impactful, actionable strategies that address the needs of both national and regional agriculture. As a leading investigator, he has pioneered and implemented innovative programs that have contributed significantly to the development of new products, enhancing agricultural outcomes in Pakistan. Dr. Nasir's expertise extends to aligning research and operational efficiencies with the evolving challenges of the agricultural sector, leveraging skilled resource planning and process improvements to drive success.

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