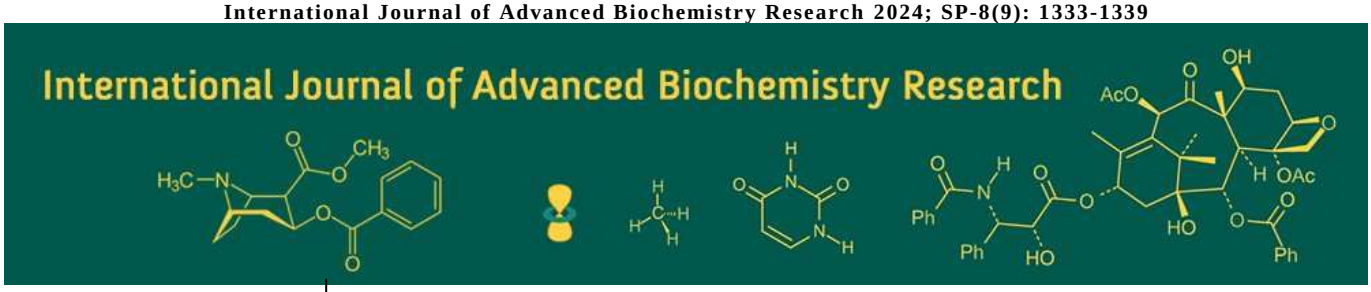

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Patel Supriya
Acharya N.G. Ranga Agricultural University, Tirupati, Andhra Pradesh, India

Aavula Naveen
Indian Agricultural Research Institute, New Delhi, Delhi, India

Dharavath Hathiram
Professor Jayashankar Telangana State Agricultural University, Hyderabad, Telangana, India

Sunaina Yadav
Indian Agricultural Research Institute, New Delhi, Delhi, India

Gampa Mallesh
Indian Agricultural Research Institute, New Delhi, Delhi, India

Thalari Vasanthrao
Professor, Jayashankar Telangana State Agricultural University, Hyderabad, Telangana, India

Hemanth S
Indian Agricultural Research Institute, New Delhi, Delhi, India

Corresponding Author:
Patel Supriya
Acharya N.G. Ranga Agricultural University, Tirupati, Andhra Pradesh, India

Cutting-Edge biotechnological methods for plant disease control: A review of advanced techniques

Patel Supriya, Aavula Naveen, Dharavath Hathiram, Sunaina Yadav, Gampa Mallesh, Thalari Vasanthrao and Hemanth S

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Abstract
Plant biotechnology helps in plant disease management in many ways, such as meristem culture which helps in developing virus-free plants and genetic modification of living organisms and their multiplication through novel technologies such as genetic engineering and tissue culture. Tissue culture in conjunction with genetic engineering is helping in inserting genes and also helpful in producing transgenic plants and disease-resistant plants. The integration of genetic engineering and tissue culture techniques enables the development of plants with enhanced resistance to specific pathogens. These advanced methods allow for the precise insertion of genes that confer disease resistance, potentially reducing the need for chemical pesticides. Furthermore, the ability to rapidly propagate disease-resistant plants through tissue culture can accelerate the widespread adoption of these improved varieties in agricultural systems. CRISPR-Cas technology, known for its precision as molecular scissors, has achieved significant success in laboratory settings, while gene pyramiding is enhancing the durability of resistant genes. The use of molecular markers for resistant breeding, transgenic plants to resist the attack of pathogens, and resistant gene enrichment sequencing are also discussed. This review explores various biotechnological approaches including RNAi-based strategies, the application of molecular markers in resistance breeding, and the concept of gene pyramiding for achieving broad-spectrum disease resistance. It emphasizes how these technologies offer more precise, efficient, and environmentally friendly solutions compared to traditional methods, while also acknowledging the need for continued research and careful consideration of ecological and societal impacts in ensuring global food security.

Keywords: Tissue culture, genetic engineering, gene pyramiding, genetic modification, transgenic plants

Introduction
Biotechnology, particularly in the realm of genetic manipulation, is increasingly being applied to address the challenges presented by a growing global population and the corresponding rise in food demand. Genetic engineering, including gene editing technologies such as CRISPR-Cas9, has been applied to enhance crop yields, improve pest resistance, and boost the nutritional profile of food (Estrada, 2017; Gartland & Gartland, 2018; Mcclements, 2019; Sharma *et al.*, 2024) [19, 23, 45]. Plant breeding techniques that help develop new varieties take a long time for testing in a field, and biotechnological approaches, such as genetic engineering and tissue culture, help in inserting the genes required for resistance into plants and developing transgenic plants. As many advances have been made, we use many tissue culture techniques and genetic engineering techniques, such as r-DNA technology, to create resistance in plants, which is not possible by breeding procedures, or which takes a long time for release (Christou 2013) [13]. These innovative biotechnological solutions encompass a wide range of techniques including genetic engineering, metabolic engineering, and synthetic biology. By harnessing the power of biological systems, researchers have been able to develop novel strategies to address complex challenges in various fields, such as medicine, agriculture, and environmental conservation (Adlak *et al.*, 2023; Rame *et al.*, 2023; Schnabl *et al.*, 2002; Yeh & Fernandez, 2004) [4, 54, 58, 70]. These advancements in biotechnology have not only accelerated the development of improved crop varieties but also opened up new possibilities for creating plants with enhanced traits that were previously unattainable through traditional breeding methods.

The integration of genetic engineering and tissue culture techniques has revolutionized the field of plant biotechnology, allowing for more precise and efficient modifications to plant genomes. This rapid progress in biotechnological approaches has the potential to address global challenges such as climate change adaptation, food security and sustainable agriculture.

Tissue culture techniques

In tissue culture, we used vegetative parts for clonal propagation and formed the basis of a number of techniques that have been developed to induce genetic changes in plants (Jain & Nakhooda, 2017 and Yildiz, 2012) [30, 71].

Applications in crop improvement:

Techniques such as somatic embryogenesis, soma clonal variation, and genetic transformation are employed to introduce desirable traits into plants, which can significantly accelerate crop improvement compared to conventional breeding methods (Pandey & Pandey, 2018 and Soman *et al*., 2019) [51, 61].

Genetic mosaics and disease resistance

Progress has been made in tissue culture and new disease-resistant cultivars have been developed; the tissues of the plant species propagated are genetic mosaics with regard to many characteristics, some may show disease resistance, and some show pest resistance, so that few plants regenerated from cultured cells are better than the parent plants. Novel plants produced using this method have been used in breeding programs by plant breeders. Plant cells can be cultured in a special nutrient medium and the whole plant can be regenerated from it (Elmawla, 2014; Kumar & Loh, 2011) [18, 38]. For example, the callus derived from infected tissues in which not all cells will carry the pathogen, for example, in the case of callus-infected cells grown from cells with TMV, only 40% of the cells may contain the virus, which may be because the virus cannot compete with proliferating cells, some mutagenesis may have occurred in them, and resistance may have been acquired. Cells resistant to the virus may be present together with susceptible parent cells. Several disease-resistant plants have evolved using soma clonal variation; for example, 370 tomato plants regenerated from six calluses showed resistance to the tobacco mosaic virus. Similarly, late blight resistance in potato (Foster *et al*. 2009; Ghislain 2018) [20, 24] and bacterial blight resistance has evolved in rice. In tissue culture, the toxins produced by pathogens are used to screen the calluses for evolving disease-resistant plants; when this screening is done, toxins kill the callus, but mutant toxin-resistant calluses survive. Regenerated toxin-resistant calluses have been used to develop disease-resistant plants. For example, the host-specific toxin produced by Helminthosporium maydis is resistant to brown spot disease and resistant plants (Holden & Sze, 1987; Miller & Koeppe, 1971 and Smith & Toth, 1982) [29, 47, 60].

Meristem or Shoot tip culture

Meristem culture helps eliminate viruses from infected germplasms and produces virus-free plants. Meristem cells, which are rapidly growing cells in plants, are free from viruses or may have a low concentration of viruses when compared to non-meristem cells. Many viruses attack plants, some may show symptoms, and some may not show symptoms at all, but yield and quality may decrease due to viral attack, which can be observed in potato, and many crops in this meristem culture may help in producing virus-free plants. In virus-infected plants, the meristem may be free from viruses, so they can be grown from the meristems of potato, sweet potato, and cassava (Jemal & Feyissa, 2020; Arkorful *et al*., 2015 and Frison & Ng, 1981) [31, 8, 21].

Mechanisms underlying the resistance of meristems to viruses

1. Inhibitors present in the meristem do not allow viral growth.
2. Some substances are produced by the viruses that break down the virus.
3. It does not have any vascular connections, which aids in the exclusion of the virus.
4. It does not have all the machinery for replication of the virus, so the virus may die.
5. Competition for key metabolites occurs as they rapidly divide.

Protoplast fusion

To obtain disease resistance in plants we may see for resistance source and we cross with them and this resistant source may be closely related species or distantly related species in both cases closed related transfer of resistance genes is easier and there won`t be any barriers but crossing between distantly related species is difficult and we have to overcome barriers (Armita, 2020; Lynch *et al*., 1993) [9, 42]. In this case, protoplast fusion helps avoid crossing barriers and viable hybrids can be produced even with distantly related species.

For example, resistance to several diseases has been transferred from *Solanum demissum* to cultivated potato varieties. *Solanum demissum*, a wild potato species, is a significant source of resistance genes, particularly in combating late blight caused by *Phytophthora infestans* (Colton *et al*., 2006; Deshmukh & Howard, 1956; Zhang *et al*., 2014) [14, 16, 72]. The deployment of resistant varieties using traditional breeding methods has been a cornerstone in the management of this devastating disease (Colton *et al*., 2006) [14].

Chemically induced fusion:

Isolated protoplasts are sticky and tend to aggregate in suspension and show spontaneous fusion during incubation (Gamborg & Miller, 1971) [47]. Chemicals increase the fusion frequency (David & David, 1979) [15]. Fusion occurs in the presence of high ca2+ and PH (9-10) but. Adhesion of neighboring cells and subsequent dilution of PEG once or either stepwise results in fusion or mixing of the cytoplasm. Polyethylene glycol causes slight dehydration of protoplasts and crinkling of the membrane. The level of fusion is 1-10% as these chemical fusing agents are toxic and therefore damaging to the cell. For example, protoplasts of plants can be inoculated with viruses to study their replication and physiology.

Recombinant DNA technology

Molecular biology has opened many opportunities of identifying and isolating the gene from an organism and mobilizing and expressing it in a different organism.

Engineering plants for resistance to disease

Notable success has been achieved with regard to viral diseases following the use of recombinant DNA technology. For example, the coat protein genes of tobacco mosaic virus (Abel *et al.*, 1986) [2] and alfalfa mosaic virus were transferred and expressed in tobacco. This lead to protection against by delaying its development in transgenic plants**.**

RNA interference technique and its use in disease management: Noncoding RNA's which play a central role in the development of resistance, are known as RNA silencing (Rosa *et al.* 2018) [56]. It is recognized as an endogenous pathway for negative transcriptional regulation. RNA interference operates in both plants and animals and uses double-stranded RNA interference as a trigger that targets homologous mRNAs and degrades them or inhibits transcription and translation mechanisms. Therefore, it is used as a method of gene targeting in fungi, bacteria, viruses, and plants, as it allows many hundreds and thousands of genes to be tested (Aman *et al.*, 2018; Lindbho and Dougherty 2005) [7, 40]. Therefore, researchers are attempting to deliver active molecules that trigger the RNA interference pathway in plants. There are many methods for transferring dsRNA into cells and tissues, including transformation with dsRNA-forming vectors for selected genes using Agrobacterium-mediated methods (Du and Vleeshouwers 2014) [17].

The most reliable and commonly used method is agro-infiltration, which is a powerful method for studying processes associated with RNAi. The injection of agrobacteria carrying DNA constructs into the intercellular spaces of leaves to trigger RNA silencing is known as agroinfiltration or agroinoculation. In most cases, it is used to initiate systemic silencing or monitor the effects of suppressor genes. In this method, a tobacco rattle virus-based vector was introduced into tomato plants by infiltration delivery of dsRNA into tobacco suspension cells by a cationic oligopeptide polyarginine Si-RNA complex, which is a linear or circular template into the nucleus (Padmanabhan & Dinesh-Kumar, 2011) [38].

Synthetic siRNAS are delivered into plants by biolistic pressure, causing the silencing of GFP expression. In this method, the delivery of cognate dsRNA of uida GUS (B-glucouridinase) and TAGLP2a:GEP (green fluorescent protein) reporter gene introduced into maize, barley, wheat by particle bombardment, and virus-induced gene silencing is a post-transcriptional gene silencing (PTGS)-based technique (Rhee *et al*., 2022; Zhou *et al*., 2021) [55, 74], which exploits the natural defense mechanisms employed by plants to protect against invading viruses for VIGS, and viral genomes remove genes that induce viral symptoms and cloning of c DNA of the viral genome into binary vectors under the CaMV35s promoter along with convenient multiple cloning sites to facilitate insertion of target gene fragments (Abe *et al*., 2005; Rüth *et al.*, 1992; Pauli *et al*., 2004) [1, 57, 57]. VIGS vectors were constructed by cloning a fragment of plant target genes with efficient siRNA generation and no off-target genes into the modified viral genome. Here, modified viruses act as RNA silencing triggers and are used to induce RNA interference in plants. Various RNA and DNA viruses have been modified to serve as vectors for gene expression. Some viruses, such as tobacco mosaic virus, potato virus X, and tobacco rattle virus, can be used for both protein expression and gene silencing.

Despite substantial advances in plant disease management strategies, the global food supply remains threatened by many pathogens and pests. This changed scenario forces us to respond quickly and effectively to provide efficient solutions to all problems. In this situation, we have combined conventional, nonconventional, and modern technologies. In this sense, RNA interference technology helps to build up resistance against fungi, viruses and bacteria which causes huge losses to agricultural crops.

Table 1: Pathogens, targeted regions, and their effects on disease management

Pathogen	Targeted region	Result
Magnaporthae oryzae	eGFP	Sequence-specific degradation of mRNA
Cladosporium fulvum	cgl 1 and cgl2	Blocking disease infection spread
Blumeria graminis	Mlo	Immunity

Use of transgenics in plant disease management:

Disease resistance genes could be obtained from plant pathogens itself, as well as coat proteins responsible for viral resistance and with toxin inactivating protein mediated bacterial resistance (Lindbo and Falk 2017) [41]. Host plants also contribute number of disease resistance genes like pathogenesis related proteins which are produced against fungal diseases.

Candidate genes found against viral pathogens:

One successful example is the management of papaya ring spot virus in Hawaii (Gonsalves 1998; Gonsalves 2006 and Ye and Li, 2010) [25-26]. Traditional breeding procedures are not able to develop resistant varieties, as there are many barriers for crossing varieties to develop new ones. The coat protein obtained from the Hawaii source was expressed in transgenic plants, and one of the coat proteins showed resistance against these viruses. Recently, gene silencing mechanisms have been put to productive use in the rice yellow mottle virus. The reading frame of the virus was expressed in rice to stop the spread of the virus. Similar attempts have been made to contain multiple viral infections (tomato spotted wilt and turnip mosaic viruses).

Candidate genes found against bacterial pathogens:

Xa-21, a broad-spectrum bacterial blight resistance gene obtained from source African rice, *Oryza longistaminata*, was backcrossed to the cultivated variety by scientists of IRRI (Halterman *et al.* 2008) [28]. The resistance gene was cloned using molecular means by Pam Ronald of the University of California and distributed to all laboratories worldwide, so that the gene could be put into rice cultivars of local importance.

Candidate genes responsible for fungal resistance

Pathogenesis-related proteins play a key role in defense by attacking and degrading the pathogen cell wall components. It has been found that typical candidate genes encoding chitinases and β-glucanases, which increase the expression of individual and multiple pathogenesis-related proteins in various crops, have been successful in enhancing disease resistance against particular pathogens [for example, in rice against *Rhizoctonia solani*, the sheath blight pathogen

(Naseri, 2012)] [8]. Research has shown that chitinases from anti-fungal bio-control species such as *Trichoderma viridae* confer transgenic resistance against rice sheath blight pathogens.

Making use of CRISPR-Cas to target viral pathogens directly: In the recent past, CRISPR-Cas (Clustered regularly interspaced short palindromic repeats) technology derived from bacteria has shown great potential for the development of resistance against plant viruses. The specificity of the cleavage depends on the presence of base complementarity between the CRISPR RNA and the target DNA or RNA (Ali *et al.,* 2015; MCGinn and Marraffini, 2019) [5, 46]. Several Cas proteins with sequence-specific nuclease activity have been identified, including the RNA guide endonuclease Cas9 from *Streptococcus pyogenes* (SpCas9) which causes double-stranded breaks in DNA *in vivo*, and the RNA-guided RNases Cas13a from *Leptotrichia wadei* which targets RNA *in vivo* (Abudayyeh *et al*., 2017) [3]. FnCas9 from *Francisella novicida* (Ginn and Marraffini, 2019) [46] is another example of a Cas protein that can cleave both DNA and RNA *in vivo*. The CRISPR Cas platforms based on CAS9 or CAS13a have shown successful use in engineering resistance to DNA viruses or RNA viruses in plants (Jupe *et al.,* 2015; Jupe *et al.,* 2013 and Ali *et al*., 2015) [15, 14].

For example, tomato yellow leaf curl DNA contains single-stranded circular DNA genomes (Mahas and Mahfouz 2018) [43]. The viral genome forms double-stranded intermediates during replication inside the host cell nuclei. Over expression of Sp Cas9 and artificially designed guided RNA's targeting the various regions of TYLCV (Tomato yellow leaf curl virus) conferred resistance to the virus in *Nicotiana benthamiana* and tomato (Tashkandi *et al.,* 2017; Tashkandi *et al.,* 2018; Bendahmane and Gronenborn 1997) [64, 65]. FnCas9 has been used to engineer resistance against RNA viruses, such as cucumber mosaic virus and tobacco mosaic virus, in *Nicotiana benthamiana* and Arabidopsis thaliana (Zhang *et al.,* 2018) [73]. Although a field test of CRISPR-Cas antiviral resistance in crop species has not yet been reported, laboratory studies have demonstrated its potential as an antiviral tool.

Resistant gene enrichment sequencing

Therefore, we need to have nucleotide-binding rich repeat (NLR) gene family numbers as they play a major role in disease resistance. In this sequencing, we used NLR gene-specific DNA fragments acts as bait to capture and enrich NLR gene-specific DNA fragments from resistant germplasms. NLR encoding DNA is sequenced and then compared with the reference genome, so that we can identify polymorphisms in NLR genes that have detected disease resistance in the resistant germplasm of interest.

For example, RenSeq was effective in accelerating the detection and cloning of the anti-*Phytophthora infestans* NLR gene Rpi-amr3i obtained from wild relatives of potato (Solanum americanum) (Witek *et al.,* 2016 and Jupe *et al*., 2014) [69, 15]. The transgenic expression of Rpi-amr3i in potato given full resistance to the late blight pathogen under greenhouse conditions.

Application of molecular markers for resistance breeding: Different types of molecular markers (DNA-based markers) have been developed and used to develop resistant rice varieties. This application of markers for breeding disease-resistant varieties is especially interesting for resistance traits that are difficult to obtain and expensive so that we cannot access them phenotypically. For example, resistance to Turcicum leaf blight in sorghum crop accession G-118 segregates as a single dominant trait when it is crossed with the susceptible cultivar HC-136 (Beshir *et al*., 2016) [11]. Therefore, to identify a molecular marker linked to Turcicum leaf blight resistance, SSR markers coupled with bulk segregant analysis were used. In the same population, an SSR marker and Xtxp 309 produced amplification of a 450 bp band. This was found to be located 3.12 cM away from locus governing resistance to leaf blight, so this was considered to be closely linked than 7.5 cM away from the locus governing the susceptibility to leaf blight, so by running the marker we identified where resistance is located (Beshir *et al*., 2016) [11].

Table 2: Application of marker assisted selection in some crops

Character/Trait	Target gene	Type of marker used
Bacterial blight in rice	xa5, xa13 and Xa21	CAPS for xa5(RG556+Dral), CAPS for xa13(RG136+Hinfl), STS for Xa21(pTA248)
Blast resistance in rice	Pi-9(t)	pB8

Gene pyramiding for broad spectrum disease management:

From the olden days onwards, breeders would identify a resistant variety and then cross it with other susceptible varieties to transfer resistance genes to develop and release resistant varieties. After some time Avr genes become virulent and this situation is vulnerable to the crop again then crop breeders had to develop resistant variety, again this resistance is lost due to two reasons, first reason is one pathogen genes are gaining resistance *i.e.,* Avr gene becoming virulent and second reason is durability of R (resistant) gene to show resistance. The adaptation of pathogens to the loss of Avr gene function can lead to virulence, as seen in *Xanthomonas oryzae pv. oryzae* (Xoo) adapted to rice R genes, where mutations at the 3' terminus of the avrXa7 allele were associated with reduced aggressiveness (Cruz *et al.,* 2000) [68]. Similarly, the breakdown of the Rlm7 resistance gene in *Brassica napus* due to repeat-induced point (RIP) mutations at the AvrLm4-7 locus demonstrates pathogen adaptation (Wouw *et al.,* 2022) [67]. Interestingly, the durability of the R genes is influenced by the cost of pathogen adaptation.

For instance, the Xa7 gene in rice was found to be durable because its adaptation to Xoo was associated with a fitness penalty (Cruz *et al*., 2000) [68]. In contrast, the avr gene avrXa27 from Xoo and its corresponding R gene Xa27 in rice are differentially expressed in the presence of the AvrXa27 effector, suggesting a complex interaction that could affect durability (Gu *et al.,* 2005).

The complexity of R-Avr interactions is further highlighted by the fact that some Avr genes are recognized by multiple R genes and vice versa, which can influence the evolution of both R and Avr genes and their management in agriculture (Petit-Houdenot & Fudal, 2017) [54]. Another way to stop

gene pyramiding is to use it as an alternative to single gene deployment so that multiple R genes can be bred into individual plant lines (Laroche *et al.*, 2019; Thorat *et al.*, 2024) [39, 66]. We used them transgenically, that is, a single R gene that was previously proven to be durable.

For example, the pepper gene Bs2 provides long-standing resistance against bacterial spot disease (*Xanthomonas campestris*). Bs2 has been cloned from pepper and shown to encode the NB-LRR protein (Tai *et al*., 1999). Xanthomonas campestris is also a significant pathogen of tomato and pepper, Bs 2 transgene works effectively against *Xanthomonas campestris* in tomato (Kearney & Staskawicz, 1990; Kim *et al*., 2010) [63, 37]. Recently cloned genes with potential use against pathogens include barley Rpg1 and tomato Ve1 and Ve2. Rpg1 has provided durable resistance to stem rust for decades, while Ve1 and Ve2 provide resistance to different verticillium species, which are functional in potatoes when expressed as transgenes (Castroverde *et al*., 2017; Kawchuk *et al.*, 1994; Nazar *et al*., 2018; Nazar *et al.*, 2019) [12, 35, 50].

Conclusion

Several biotechnological methods and tools have emerged in recent years and are becoming effective in disease management. Among these successful biotechnological tools, tissue culture techniques have been widely adopted by many countries in the early stages to produce disease-free plants. Later, with the introduction of new technologies in biotechnology, such as the use of molecular tools for disease management, it has become very simple and quick, which saves time. Some highly appreciable modern techniques such as CRISPR-Cas, Renseq, Durable resistance via gene pyramiding for broad-spectrum disease management as well as RNAi (gene silencing) technology and genetic engineering techniques such as agroinfiltration, VIGS, and micro bombardment have proven to be viable technologies for disease management. These technologies are economical, eco-friendly, and require less time to develop resistant varieties when compared to conventional breeding techniques that take a longer time.

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