



CURRENT ISSUES IN PLANT DISEASE CONTROL: BIOTECHNOLOGY AND PLANT DISEASE

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ABSTRACT

Despite substantial advances in plant disease management strategies, our global food supply is still threatened by a multitude of pathogens and pests. This changed scenario warrants us to respond more efficiently and effectively to this problem. The situation demands judicious blending of conventional, unconventional and frontier technologies. Biotechnological procedures can be used to determine the type and sources of host resistance. Biotechnology is the genetic manipulation and multiplication of any living organism through novel techniques and technologies such as tissue culture and genetic engineering in order to produce new organisms and or products that can be used in variety of ways. It is theoretically possible to express virtually any genetic trait of an organism in plants. The development of recombinant DNA technology makes it possible to isolate individual genes and incorporate resistance genes into otherwise agronomically acceptable cultivars. Genes pyramiding was made easier with molecular markers. ELISA and polymerase chain reaction (PCR) techniques are used in the identification of viral and bacterial disease and also new formats using antibody based detection for very rapid presumptive on-site diagnosis have become available. These do not require specialized equipment or knowledge. Most of them use a membrane based lateral flow assay in which capillary forces generate a migration of the sample extract over specific antibodies. The aim of this write up is to review the major application of biotechnology to the control of plant diseases.

Keywords; *Biotechnology, tissue culture and genetic engineering, plant disease management strategies,*

INTRODUCTION

According to Agrios (1988; 2005) biotechnology is the genetic manipulation, and multiplication of any living organism through novel techniques and technologies such as tissue culture and genetic engineering resulting in the production of improved or new organism and products that can be used in variety of ways. Traditional plant breeding methods have been used to develop cultivars resistant to various diseases (Agnihotri *et al.*, 1989). But this process is time consuming and limited availability of genetic resources for most of the crops are available and has left little room to continued improvement by this means. Development of crop varieties which are resistant against many economically important diseases is a major challenge for plant biotechnologists, worldwide. Plant diseases are a threat to world agriculture and general food security. Significant yield losses due to the attack of pathogen occur in most of the agricultural and horticultural crop species. Example; in Nigeria 25 million Naira was lost when about 70 % of the cocoa produced was lost to black pod disease in 1995 (Kutama *et al.*, 2011a,b). More than 75% of all major crops diseases are caused by fungi (Kutama, 2012). Traditional plant breeding methods have been used to develop cultivars resistant to various diseases. However, this process is time consuming and limited availability of genetic resources for most of the crops has left little room to continued improvement by these means (Mehrotra and Aggarwal, 2003). Two most important reasons for limited genetic resources available for breeding are that many of the natural

gene traits that may be beneficial in one plant tissue may be deleterious in other plant tissue and that loss of genes pools recurring during the domestication and breeding of crop plant (Cook and Baker, 1983). Modern technologies such as transcriptomics, proteomics and metabolomics are now proved to be useful in understanding plant metabolic pathways and the role of key genes associated with their regulation. This can facilitate new insights into the complex metabolite neighborhoods that give rise to a given phenotype and may allow discovery of new target genes to modify a given pathway. Such genes can then be subject to new metabolic engineering efforts and applications. It has become routine to transfer genes from one organism to another, genes conferring disease resistance to crop plants have been introduced. Such gene transfers could be accomplished by direct methods: e.g. the gene or biolistic method and agrobacterium mediated method. Vector mediated method (Agnihotri *et al.*, 1989). Biotechnology permits accurate diagnosis of plant disease. ELISA and PCR techniques are used in the identification of viral and bacterial diseases. The aim of this write up is to review the major application of biotechnology to the control of plant diseases.

Role of Biotechnology in Plant Disease Control

Recent advances in plant genetic engineering strategies for the management of bacterial diseases of plants are now available. Genetic engineering for plant disease resistance has been discussed by many workers.

The use of tissue culture and genetic engineering for controlling plant diseases has been recently reviewed by Fuchs and Gonsalves (1996), while the role of biotechnology in controlling plant disease has been discussed by Mandahar and Khurana (1998).

Plant biotechnology impinges or helps plant pathology in many ways;

1. To obtain pathogen-free mother plants through rapid clonal propagation.
2. New plants to which genes have been incorporated through genetic engineering are likely to show instability towards environmental conditions and towards the pathogenic microflora of their habitats. Here, pathology plays its part (Gilchrist, 1998).
3. The main vehicle for transferring genes from donor to recipient, in plant pathogens, particularly the bacterium *Agrobacterium tumefaciens* and the cauliflower mosaic virus.
4. Control of plant diseases by inserting resistance genes into plants by genetic engineering techniques.
5. The study of plants genes for resistance to disease and of pathogen genes for virulence

to pathogen has already added considerably by genetic engineering techniques.

Tissue Culture Techniques

Almost all tissue culture techniques are used in plant pathology. Some of the importance tissue culture techniques and their importance to plant pathology are briefly described;

Protoplast Fusion;

Disease resistance in breeding program may come either from closely related species or from more distantly related species. Problems are generally encountered if an effort is made in crossing distantly related species. Protoplast fusion is one of the methods that can be used to circumvent problems in introgression genes for resistance. By this method, factors that contribute to crossing barriers between species can be avoided and viable hybrids (Cybrids) have been recovered even between distantly related species (Harms, 1985). Examples of disease resistant plants, produced from protoplast fusion are shown in Table 1 below;

Table 1: Disease Resistant Plants Produced from Protoplast Fusion

Species used for fusion	Diseases	Reference
<i>Lactuca sativa</i>	Dowmy mildew (<i>Bremia lactucae</i>)	Maloy, (2005)
<i>Brassila oleracea</i> and <i>Raphanus sativus</i>	Club root (<i>Plasmodiophora brassical</i>)	Maloy, (2005)
<i>Brassica napus</i> and <i>Brassica nigra</i>	Black leg (<i>Phoma lingam</i>) club root.	Maloy, (2005)
<i>Solanum brevidens</i> and <i>Solanum tuberosum</i>	Bacterial soft rot (<i>Erwinia</i> spp)	Maloy, (2005)

Source: (Eck and Smith, 1996).

Chemically induced fusion;

Isolated protoplasts are sticky, tend to aggregate in suspension and show fusion spontaneously during incubation. Chemicals tend to increase the fusion frequency. Fusion can occur in the presence of high Ca^{2+} and high pH (9-10) but a commonly used chemical (Fusogen) is polyethyleneglycol (PEG). Due to the addition of PEG there is adhesion of protoplast to their neighbors which can be assessed by microscope. Subsequent dilution of stabilized PEG, either stepwise or at once results in fusion and mixing of the cytoplasm. PEG causes slight dehydration of the protoplasts and crinkling of the membrane. The level of fusion is usually 1-10% as chemical fusion agents are toxic and therefore damaging to the cell (Zimmerman and Scheurich, 1981).

It has been possible to inoculate the protoplast of plant with viruses and study their replication and physiology. The bacterium *Agrobacterium tumefaciens* or its modified T-plasmid and the double-stranded DNA virus cauliflower mosaic virus have been used to introduce foreign genetic material into plant cell.

Selection for Disease Resistance:

In-vitro selection has a distinct advantage over other selection systems since it allows significant saving of space, time and money. For plant diseases that cause damage through toxins, cell selection for toxin resistance in cultures and regeneration of plants from

descendants of the selected cell lines can give disease-resistant genotype. For example, disease resistant crop plants have been produced through in vitro selection in potato against *Phytophthora infestans* (late blight of potato), in tobacco, (*Nicotiana tabacum*) against *Pseudomonas tabli*. At the biotechnology centre, IARI, new Delhi, plants resistant to toxin produced by *Alternaria brassica*, a blight causing organism, have been isolated (Nagarajan *et al.*, 1992).

Recombinant DNA Technology:

Advances in molecular biology have opened up possibilities of identifying and isolating any gene for an organism, and mobilizing and expressing it in a different organism of one's choice.

(i) Engineering Plants for Resistance to Disease;

A notable success has been made with regard to viral diseases following use of r-DNA technology (Maloy, 2005). For example, a major achievement has been the transfer and expression of coat protein genes of tobacco mosaic virus (TMV) and alfalfa mosaic virus (AMV) in tobacco, resulting in protection against or delay of disease development in the transgenic plants (Beachy *et al.*, 1990). The purpose of introducing coat protein genes to give resistance against the virus is that the multiplication of infecting viral RNA is somehow checked by coat protein synthesized in the plant cells.

Engineered plants synthesized chitinase which breaks down the fungal cell wall and this kills the soil borne pathogen, *Rhizoctonia solani* (Maloy, 2005; Schell and Vasil, 1989).

(ii) Engineering Plants for Resistance to Pest

The best way of insect control has been the use of insecticides. These insecticides were effective but proved to be environmental hazards and forced development of resistant strains of insects. There are genes in bacteria (*Bacillus thuringiensis*) that encode insecticidal proteins. *Bacillus thuringiensis* strains toxic to dipteran, lepidopteran, and coleopteran insects have been identified and the insecticidal protein gene cloned. Using T plasmid vectors of *Agrobacterium tumefaciens*, the gene encoding the insecticidal protein has been transferred to tobacco, potato, tomato, rice and corn. Such transgenic plants incorporate resistance to specific insects that feed on these crops (Schell and Vasil, 1989).

RNA-interference Technique

During the last decade, RNA-mediated functions has been greatly increased with the discovery of small non-coding RNAs which play a central part in process called RNA silencing. Ironically, the very important phenomenon of co-suppression has recently been recognized as a manifestation of RNA interference (RNAi), an endogenous pathway for negative post-transcriptional regulation. RNAi has revolutionized the possibilities for creating custom "Knock down" of the gene activity. RNAi operates in both plants and animals, and use double stranded RNAi (dsRNA) as a trigger that targets homologous mRNAs for degradation or inhibiting its transcription translation (Maloy, 2005). It has been emerged as a method of choice for gene targeting in fungi (Maloy, 2005), viruses, bacteria and plants as it allows the study of the function of hundreds of thousands of genes to be tested (Mehrotra and Aggarwal, 2003).

Mechanism of RNAi

RNA interference refers collectively to diverse RNA based processes that all result in sequence-specific inhibition of gene expression at the transcription, mRNA stability or translational level. The unifying features of this phenomena are the production of small RNAs (21-26 nucleotides (nt)) that act as specific determinants for down-regulating gene expression (Issac,1992) and the requirement for one or more members of Argonaute family of protein (Maloy, 2005). RNAi operates by triggering the action of dsRNA intermediates, which are processed into RNA duplexes of 21-24 nucleotides by a ribonuclease III-like enzyme called Dicer (Mehrotra and Aggarwal, 2003). Once produced, these small RNA molecules or short interfering RNAs (siRNAs) are incorporated in a multi-subunit complex called RNA induced silencing complex (RISC) (Mehrotra and Aggarwal, 2003): RISC is formed by a siRNA and an endonuclease among other component. The siRNAs within RISC acts as a guide to target the degradation of complementary messenger RNAs (mRNAs), (Balasubramanian, 2009). When dsRNA molecules produced during viral replication trigger gene silencing, the process is called

virus-induced gene silencing (VGS) (Maloy, 2005). One interesting feature of RNA silencing in plants is that once it is triggered in a certain cell, a mobile signal is produced and spread through the whole plant causing the entire plant to be silenced. This silencing process is also enhanced by the enzymatic activity of the RISC complex, mediating multiple turnover reaction (Broglie *et al.*, 1991).

Furthermore, production of the secondary siRNAs leads to enrichment of silencing via its spread from the first activated cell to neighboring cells, and systematically through system (Maloy, 2005). The cell-to-cell spread can be mediated as passive spread of the small RNAs via plasmodesmata, since it does not spread into meristematic cells (Balasubramanian, 2009). The discovery of RNA binding protein (PSRPI) in the phloem and its stability to build 25 ntssRNA species add further to the argument that siRNAs (24-26nt) are the key components for systemic silencing signal (Brain and Beathle, 2003)).

Methods of Inducing RNAi in Plants:

One of the biggest challenges in RNAi research is the delivery of the active molecules that will trigger the RNAi pathway in plants. In this system, a number of methods for delivery of dsRNA or siRNA into different cells and tissue include transformation with dsRNA forming vectors for selected gene(s) by an *Agrobacterium* mediated transformation (Balasubramanian, 2009). delivery cognate dsRNA of uidA GUS (B-glucuronidase) and TaGLP2a: GEP (green fluorescent protein) reporter gene into single epidermal cells of maize, barley and wheat by particle bombardment (Broglie *et al.*,1991), introducing a *Tabacco rattle virus* (TRV) based vector into tomato plants by infiltration delivery of dsRNA into tobacco suspension cells by cationic oligopeptide polyarginine-siRNA complex; infecting plants with viral victors that produce dsRNA (Dalmay *et al.*, 2000) and delivery of siRNA cultured plant cells of rice, cotton and slash pine for gene silencing by Nano sense pulsed laser-induced stress wave (LISW) (Maloy, 2005).Among these, the most reliable and commonly used approaches for delivery of dsRNA to plants cells are agro-infiltration, micro-bombardment and VIGS (Maloy, 2005).

Disease Management in RNAi;

In this sense, RNAi technology has emerged as one of the most potential and promising strategies for enhancing the building of resistance in plants to combat various fungal, bacteria, viral and nematode diseases causing huge losses in important agricultural crops (Singh, 2005).

The nature of this biological phenomenon has been evaluated in a number of host-pathogen systems and effectively used to silence the action of pathogen. Many of the examples listed below illustrate the possibilities for commercial exploitation of this inherent biological mechanism to generate disease-resistant plants in the future by taking advantage of this approach e.g. including; *Cladosporium fulvum* (Singh, 2001)) *Magnaporthe oryzae*, *Venturia inaequalis* and *Neurospora crassa* (Singh, 2005).

Monoclonal Antibodies Technique

The hybridoma technique was developed by George Kohler and Cesar Milstein in 1975 at the Medical Research Council Laboratory in Cambridge, England. This technique was never patented but its commercial applications were recognized immediately. This discovery led to the production of monoclonal antibodies (Mandahar and Kurana, 1998). In this technique there is the fusion of myeloma cells (cancer cells) with antibody-producing white blood cells (B-lymphocytes). The resulting hybrid cell is called a hybridoma (Balasubramania, 2009).

In the last few years, techniques have been developed to produce large quantities of identical antibodies. These new antibody-forming hybrid cells, hybridomas, can now be grown in culture indefinitely. Each hybridoma clone produces only one type of antibody. But via selection techniques the clone that produces the desired antibody can be chosen. Monoclonal antibodies can be obtained from the liquid of hybridoma cultures and can be used to detect, identify and measure the antigens that induced their production (Nagarajan, *et al.*, 1992).

Monoclonal antibodies are however, very specific and may not detect strains of the same virus. It is for this reason that mixtures of several monoclonal antibodies are often used in the detection of viruses and in screening tests. The various techniques involved are:

1. Rats are immunized by injecting the selected antigen either subcutaneously or into the peritoneal cavity.
2. A few days later, the spleen is removed from the rat.
3. The antibody synthesizing cells from the spleen are then mixed with fast growing rat cancer cells, called myelomas in the presence of polyethylene glycol (PEG).
4. The result is hybridoma-fusion of an antibody-making cell and a cancer cell.
5. All the hybridoma cells that occurred after a fusion were grown in selective culture medium, then a mixture of antibodies would be released.
6. Individual hybridoma cells are grown as a clone. In this way, only one type of antibody will be secreted by all the cells in a clone.
7. After the desired hybridoma cell lines have been identified and cloned, culture may be expanded and clones may be tested for the desired antibody.
8. Once the cell has been cloned, either selected clones are cultured and frozen or the hybridoma cell may be injected into the peritoneal cavity of a rat, to produce ascetic fluid.
9. From the ascetic fluid, monoclonal antibodies are purified.
10. The stored and frozen cells can be used at different times of the year and in different laboratories (Maloy, 2003).

Uses and Applications of Monoclonal Antibodies

1. The most effective application of monoclonal antibodies has been with plant pathogenic viruses. This technique has been used for accurate identification of viruses for tracing the viruses during epidemiological studies, to distinguish between virus

strains and in isolation and purification of viruses (Chopra and Sharma, 1991).

2. These procedures are now being used to study bacteria and fungi and will probably be used in investigation of various populations of rhizosphere organisms. This technology is also very helpful in attempts to identify bacteria in mixed populations. Application of this technology to fungi is in its infancy, but the potential is great (Chopra and Sharma, 1991).

Transgenic Plant Disease Management:

Diseased resistance genes could be sourced from plant pathogens themselves, as was possible with coat protein-mediated plant viral resistance and with toxin-inactivating protein-mediated bacterial resistance (Agrios, 1988). Host plants also contribute an enormous number of disease resistance genes such as those encoding pathogenesis-related (PR) proteins, which have been used against fungal disease (Schippers, 1983).

Candidate Genes against Viral Pathogens

One of the most successful examples, as of date of the use of transgenic resistance against plant disease is that which was accomplished in the management of papaya ring spot virus (PRSV) in Hawaii (Jain, 1993). Traditional breeding in bringing about resistance against this disease was of no avail as crossability barriers were a big problem. Under these circumstances, coat-protein-mediated resistance using coat protein genes sourced from a Hawaiian strain of PRSV was attempted. One transgenic line was found to be completely resistant to PRSV (Mandahar and Khurana, 1998; Balasubramania (2009)).

Transgenic resistance against banana bunchy top resistance using a BBTV replicate gene is under study. However, it might take some more time to be successful in this attempt, as we have yet to accomplish much in the routine generation of transgenic banana lines of local importance (Agrios, 2005). Once generation of transgenic banana has become routine, it will be easier to deliver human vaccines (cholera toxin vaccine and hepatitis B surface antigen) via transgenic banana fruits, as banana fruit forms an excellent delivery material (Jain, 1993).

Recently, a gene silencing mechanism has been put to productive use in obtaining rice yellow mottle virus. An open reading frame of the virus itself is expressed in rice in order to stop the viral spread in an effective manner. Similar attempts also have been made in obtaining multiple viral infections (tomato spotted wilt virus and turnip mosaic virus) in plants (Chopra and Sharma, 1991).

Candidate Genes against Bacterial Pathogens

A wide-spectrum bacterial blight resistance gene Xa21, sourced from an African rice, *Oryzalongistaminata* was backcrossed into cultivated variety by scientists of the International Rice Research Institute, the Philippines (IRRI). The resistance gene was cloned using molecular means by Pam Ronald of University of California and distributed to labs all over the world, so that the gene could be put into rice cultivars of local importance (Oswald, 1951).

Wild Fire disease of tobacco caused *Pseudomonas syringae* PV. *tabaci* is a serious disease. A phytotoxin secreted by the pathogen drastically modifies the amino acid metabolism of the plant with the eventual accumulation of ammonia in tobacco leaves, which causes extensive blighting (Maloy, 2003). Interestingly, the pathogen that synthesises the phytotoxin remains unaffected by the toxin. This formed the basis for a search of the candidate gene from the pathogen itself. A toxin-inactivating gene, which was named 'tr' was successfully isolated from the pathogen and the same was cloned into tobacco cultivars, which showed excellent wildfire resistance (Agrios, 1988).

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