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MOLECULAR MARKERS AND DISEASE RESISTANCE IN PIGEONPEA

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Abstract: *Fusarium wilt, Sterility mosaic and Phytophthora stem blight are important diseases of pigeonpea which causes huge yield losses. Till now various resistant varieties have been developed against these diseases but very little is known about the genetics and marker assisted selection in pigeonpea. The present review is an effort to compile most of the research works done by several workers in terms of genetics of disease resistance and molecular markers.*
Keywords: *Cajanus cajan, molecular markers, genetics, disease resistance.*

Introduction: *Cajanus cajan* or pigeonpea, is a diploid (2n = 22), often cross-pollinated crop with a genome size of 858 Mbp [1]. It is predominantly cultivated as wet season crop in the tropical and subtropical regions of Asia and Africa [2]. In India, it is cultivated in Maharashtra, Karnataka, Andhra Pradesh, Uttar Pradesh, Madhya Pradesh, Gujarat and account for over 70% of the total pigeonpea cultivating area [2]. It is a rich source of protein, minerals and vitamins; hence, it plays an important role in food and nutritional security. Knowledge of the genetic basis of yield, resistance to diseases and insect-pests and abiotic stress tolerance are important factors for deciding the breeding strategies for genetic improvement of pigeonpea. However, in comparison to other economically important crops, relatively less effort has been invested in understanding the genetics of important agronomic traits of pigeonpea.

Disease Resistant Varieties of Pigeonpea: Fusarium wilt is a very important biotic constraint to pigeonpea production. *Fusarium udum* Butler (teleomorph- *Gibberella indica*) causes wilt disease in pigeonpea, the fungus infects the plants at any stage of development and disease results in over 50% or even up to 100% loss in grain yield [3]. Sterility mosaic disease which is commonly known as 'green plague' is another serious constraint of pigeonpea production. It is a viral disease, caused by Pigeonpea sterility mosaic virus (PPSMV). At <45-day-old plants, SMD infection results in 95 to 100% yield loss, while at late plant stage (>45-day-old plants) infection depends on the level of infection (i.e., number of affected branches per plant) and range from 26 to 97%. After wilt and sterility mosaic, Phytophthora stem blight (PSB) is the third potentially important disease of pigeonpea in India, especially in north-eastern India. The disease incidence varied between 5.0-26.5% on various cultivars [4]. Besides these diseases, several other fungal, bacterial and viral diseases are also known to infect pigeonpea plants at various developmental stages. Deployment of host plant resistance is the most effective, economical and environment friendly option for management of pigeonpea diseases. Disease resistance has proved useful in controlling many diseases of edible legumes [5], but much more remains to be done in development of multiple disease and pest-resistant cultivars of pigeonpea. More emphasis should be given to host plant resistance as costs of other control measures that depend heavily on the use of fossil fuel energy and other scarce resources rise. This method of control requires that researchers have access to a large, variable source of germplasm of different food legumes to use in their breeding programs, along with the availability of recent tools and techniques. For the development of resistant varieties in many crops, Gene for gene hypothesis given by Flor have been used, which suggest that many plant

species and their pathogens follow a gene-for-gene relationship and plant disease resistance is often controlled by Mendelian genes [6]. According to this theory, there are many resistance (R) genes in a plant species against each of its pathogens and there is a corresponding avirulence gene in the pathogen population for every R gene in the host plant. This theory has been well demonstrated and utilized in development of resistant varieties, in plants where resistance is associated with hypersensitivity. However, a clear-cut resistant phenotype like hypersensitivity does not always exist in many other cases and plant resistance often shows both qualitative and quantitative components. The qualitative resistance in many plant-pathogen relationships is hypersensitive, race specific, and governed by interactions between avirulence genes in pathogens and resistance genes in hosts, while the quantitative resistance is non-hypersensitive, presumably non-race specific, and controlled by polygenes [7]. For the development of resistant varieties, the knowledge of genetics and mechanism of disease resistance and host pathogen interaction is needed. This helps in the proper deployment of host resistance. Pigeonpea is an often self pollinated, partially outcrossed crop, and the resistance breeding methods generally recommended for self pollinated crops are used for it. Pedigree selection, pure line breeding, population breeding, mutation breeding, and wide hybridization have been used for development of new varieties in pigeonpea and have led to incremental improvements in the yield potential of this crop [1]. The limited natural outcrossing has been successfully exploited for increasing yield and stability through the development of commercial hybrids using genetic male sterility. Cytoplasmic male sterility (CMS) has become a powerful method to develop and commercialize hybrids. With the advent of genomic tools such as molecular markers, genetic maps, etc., conventional plant breeding has been facilitated greatly and improved genotypes/ varieties with enhanced resistance/ tolerance to biotic/abiotic stresses have been developed in several crop species. Although a large number of germplasm lines have been identified for resistance to *Fusarium* wilt, sterility mosaic and *Phytophthora* stem blight, resistance for these diseases has been only partial and germplasm with absolute resistance is rarely available [8]. A number of resistant varieties against many diseases of pigeonpea have been developed and released by ICRISAT and other institutes for commercial cultivation (Table 1).

Table 1. List of Resistant Varieties of Pigeonpea

Disease	Resistant varieties/lines
Fusarium wilt	AL 1, BDN 2, Birsa Arhar1, DL 82, H 76-11, H 76-44, H 76-51, H 76-65, ICP 8863 (Maruti), ICP 9145, ICPL 267, Mukta
Sterility mosaic	Prabhat, Sharda, TT 5, TT 6 Bageshwari, Bahar, DA 11, DA 13, ICPL 86, ICPL 146, ICPL 87051, MA 165, MA166, PDA 2, PDA10, Rampur Rahar
Phytophthora blight	Hy 4, ICPL 150, ICPL 288, ICPL 304, KPBR 80-1-4, KPBR 80-2-1 (Field resistant)
Cercospora leaf spot	UC 796/1, UC 2113/1, UC 2515/2, UC 2568/1
Powdery mildew	ICP 9150, ICP 9177
Alternaria Blight	DA 2, MA 128-1, MA128-2, 20-105 (West Bengal)
Dry root rot	ICPLs 86005, 86020, 87105, 91028
Bacterial leaf spot and Stem canker	ICPs 12807, 12848, 12849, 12937, 13051, 13116, 13148
Phoma stem canker	AL 133, AL 136, ICPL 148, ICPL 84018
Rust	Blanco, Todo Tempo No.17
Phyllody	BDN 5, ICPL 83057, MRG 66
Halo blight	GW 3, ICPL 362
Phyllosticta leaf spot	EMC, ICPL 161, ICPL 269, ICPL 335, Pusa 33, Pusa85
Root-knot and Dirty root	ICP 11289, ICP 11299, AGS 522, Basant, GAUT 82-75, GAUT 83-23, GAUT 84-22, ICP 12744, PDM1

Molecular Markers and Genetic Improvement of Pigeonpea: Biotechnological methods can contribute significantly to the genetic improvement of pigeonpea. Plant breeders have become increasingly interested in marker assisted selection for efficient and precise transfer of genes conditioning important agronomic traits. Marker Assisted Selection (MAS) is an indirect selection process where a trait of interest is chosen not based on the trait itself but on a marker (morphological, biochemical or one based on DNA/RNA variation) linked to it. Molecular markers are DNA sequence variants that can readily be detected and whose inheritance can be monitored [9]. Molecular marker technology can facilitate the precise

determination of the number, chromosomal location and individual and interactive effects of genes that control traits [10]. However, use of MAS requires detailed information on the plant genome. A basic pre-requisite for any molecular breeding program is a robust set of polymorphic markers for the species under investigation.

Recently, molecular marker technologies have become a powerful tool in crop improvement through their use in germplasm characterization and fingerprinting, genetic analysis, linkage mapping, and molecular breeding. Random amplified polymorphic DNA (RAPD) markers provide an efficient assessment of the differences in the genetic composition of related individuals [8]. Quantitative Trait Loci (QTL) is a statistical method that links two types of information phenotypic data (trait measurements) and genotypic data (usually molecular markers) in an attempt to explain the genetic basis of variation in complex traits [11]. So far, QTLs underlying different resistance phenotypes have been identified and the scientists are now backcrossing populations to generate disease resistance QTLs into farmer preferred pigeonpea varieties. In this post-genomic era, a more thorough understanding of gene expression and function can be achieved through the characterization of the products of expression, the proteins, which are essential biological determinants of plant phenotypes. Proteomics offers a continually evolving set of novel techniques to study all facets of protein structure and function. The application of proteomics in plant pathology is becoming more commonplace with techniques such as two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) being used to characterize cellular and extracellular virulence and pathogenicity factors produced by pathogens as well as to identify changes in protein levels in plant hosts upon infection by pathogenic organisms and symbiotic counterparts [12]. In case of pigeonpea, however, a very limited number of genomic tools are available so far. For instance, 140 microsatellite or simple sequence repeat (SSR) markers, 908 expressed sequence tags (ESTs), are available in pigeonpea. Significant number of unigene sequences related to proteins like kinases, phosphatases, peroxidases, ribonucleases, endochitinases, glucanases and hormones like Abscisic acid responsive (ABA) genes were identified to be differentially expressed and are known to play a vital role in defense mechanism of pigeonpea plants against Fusarium wilt, Sterility mosaic and other diseases. For example, the cell wall degrading enzymes like endochitinases (EC: 3.2.1.14) implicate a major defense mechanism against pathogen. Similarly, kinases play a major role in the plant's recognition to pathogen. For instance, chitinase protein (UniProt ID: P23472), a class of pathogenesis related (PR) proteins with bi-functional role in lysozyme/chitinase activity involved in random hydrolysation of N-aetyl-beta-D-glucosaminide-beta linkages in chitin and chitodextrins during systemic acquired resistance (SAR), was expressed at higher concentrations in FW-responsive resistant genotype ('ICPL 20102') compared to susceptible genotype ('ICP 2376'). The high expression levels of chitinase in resistant genotype indicate the effectiveness within a narrow range of pathogenesis. The protein coding for ABA-responsive protein (ABR18) (UniProt ID: Q06930), which is involved in stimulus mechanism and cell localization etc. during plant development and one of the vital roles is in defense mechanism during biotic stress signaling was identified to be expressed relatively higher in SMD-resistant pigeonpea genotype 'ICP 7035' compared to the susceptible genotype 'TTB 7'. During pathogen infection ABA inhibits the transcription of a basic b-1, 3-glucanase (EC: 3.2.1.39) that can degrade the b-1, 3- glucan callose, forming a physical barrier to viral spread through plasmodesmata. This down regulation of b-1, 3-glucanase by ABA can be termed as a resistance factor in plant pathogen interactions. Hevamine (EC: 3.2.1.14) and Leucoanthocyanidin dioxygenase (EC: 1.14.11.19) genes were found to be specifically expressed in 'ICPL 20102' 30 DAI library. This important protein hevamine represents a new class of polysaccharide-hydrolyzing (ba) 8 barrel enzyme belonging to families of plant chitinases and lysozymes, which are vital for plant defense against pathogenic bacteria and fungi.

Molecular Markers and Fusarium Wilt: The knowledge of genetic inheritance is essential for formulation of strategy on how to transfer the genes into adapted susceptible varieties. In pigeonpea, resistance to Fusarium wilt has been reported to be under the control of two complementary genes, single dominant gene, 2 genes, major genes, duplicate genes and even multiple factors and a single recessive gene [13]. Inheritance of wilt resistance showed a ratio of 9:7, with resistance being dominant, controlled by multiple factors, the presence of two

complementary genes, and a single dominant gene. Resistance to wilt showed a single gene dominant to susceptibility [4]. However, found resistance was dominant over susceptibility [14]. Apart from dominant, recessive and complementary gene action on the control of Fusarium wilt has been reported. Dominant epistatic gene interaction and a single dominant gene play a significant role in controlling resistance to wilt. Digenic and quantitative genes that are resistant to Fusarium wilt have been observed although quantitative inheritance is often influenced by environment; the resistance depends on the source of the gene. Partial resistance to Fusarium wilt was also characterized by several authors in pigeonpea cultivars. Characterized partial resistance to Fusarium wilt by root inoculation of seven pigeonpea genotypes with a virulent isolate of *Fusarium udum* [15]. The mechanisms of resistance in the genotypes appeared to be different, with genotype ICP8863, having a longer incubation period, minimum wilt index and minimum pathogen colonization as compared to other resistant genotypes (ICP9174, ICP87119 and ICP8858). Various molecular markers have been developed and used for the identification of wilt resistance in pigeonpea plants. A study conducted [16], showed differences in genetic basis of resistance in Indian and Kenya isolates and found that *Fusarium* wilt (Kiboko isolate) in pigeonpea is controlled by recessive genes; a single recessive gene in cv. ICEAP00040, which is of East African origin and duplicate recessive genes in the Indian resistant source, ICP8863. The genetic basis of resistance in the cross involving resistant Indian genotypes was elucidated by assuming a set of 2 independent loci, i.e AABB–Susceptible parent, and aabb–Resistant parent. The 9:7 ratio indicates dihybrid segregation with complementary interaction between the 2 dominant genes. Several other workers also suggested the presence of recessive genes in many plant-pathogen relationships, although most plant resistance genes have been reported to be controlled by dominant genes. Indeed, a recent study in pigeonpea identified two Random Amplified Polymorphic DNA (RAPD) markers linked to a recessive allele of a *Fusarium* wilt resistance gene [17]. Identified two RAPD markers (704 bp and 500bp linked to Fusarium wilt susceptibility) [17] using F2 populations derived from contrasting parents GS1 (susceptible), ICPL87119, and ICP8863 (resistant). Screened 108 RAPD markers to identify cytoplasmic male sterile (CMS) [8] lines derived from crosses between wild and cultivated pigeonpea. RAPD has been used to tag Fusarium wilt resistant and CMS lines of pigeonpea. The control of resistance by recessive genes suggests a greater mechanistic complexity but can be largely attributed to mutations. The *Mlo* recessive mutation, which confers broad spectrum resistance to several isolates of the fungus *Erysiphe graminis* f. sp. *hordei* in barley (*Hordeum vulgare* L.) is a good example. In case of chickpea wilt also, earlier reports suggested that resistance to Fusarium wilt in chickpea was conferred by a single recessive gene. Recent studies have shown that resistance to race 1, appears to be controlled by at least 3 independent loci. Complete resistance is obtainable from crosses involving susceptible parents (late-wilting). PCR reactions using OPGO8 random primer with genomic DNA of different pigeonpea lines resulted in identification of six resistant sources with specific amplification for resistance to wilt at 920bp. Microsatellites or Simple sequence repeats (SSRs) provide a powerful tool for genomic studies and are recommended for systematic fingerprinting of pigeonpea germplasm. However, only 140 SSR markers were available in the public domain [16]. To enable genomics-assisted breeding in this crop, the pigeonpea genomics initiative (PGI) was initiated in late 2006 by ICAR and the Government of India, with INDO-US Agricultural Knowledge Initiative (AKI) in partnership with reputed Indian and International Organizations. Till now they have developed an 11X- genome coverage bacterial artificial chromosome (BAC) library comprising of 69,120 clones, of which 50,000 clones were end sequenced to generate 87,590 BAC end sequences. 10,000 ESTs, 21,000 SSRs have been identified and 6,698 SSRs are under analysis along with 670 orthologous genes using a Golden gate SNP genotyping platform. In addition, >600 unique nucleotide binding site (NBS) domain containing NBS-LRR disease resistance homologs were cloned in pigeonpea [18]. Recently, reported generation and analysis of comprehensive resource of FW- and SMD-responsive expressed sequence tags (ESTs) [1]. They constructed a total of 16 cDNA libraries from four pigeonpea genotypes that are resistant and susceptible to FW (’ICPL 20102’ and ‘ICP 2376’) and SMD (’ICP 7035’ and ‘TTB 7’) and generated a total of 9,888 (9,468 high quality)

ESTs. This information is available in dbEST of GenBank under accession numbers GR463974 to GR473857 and GR958228 to GR958231. Considering the wilt reaction and resistance linked RAPD, SSR and other markers, it is possible to identify the new resistance sources in a short time and they can be utilized in breeding programme or for direct release of variety. A larger number of markers would still be required in future to enable MAS in pigeonpea. The construction of large-insert bacterial artificial chromosome (BAC) libraries, as has been done in chickpea, will be necessary in pigeonpea for their potential wide genome coverage. The use of more than one restriction enzyme in library construction as well as targeting longer motifs are other options likely to maximise the yield of potentially useful SSRs across the genome. With the current efforts to make DArT technology available in pigeonpea and the falling prices in DNA sequencing and SNP assays, more superior markers will undoubtedly be incorporated to complement the current efforts and enhance molecular marker technology in pigeonpea.

Molecular Markers and Sterility Mosaic Disease (SMD): The combination of breeding and high polymorphic PCR based markers permit the identification and mapping of useful molecular markers for breeding programmes. Earlier studies indicated that susceptibility to SMD is dominant over tolerance and that resistance and disease response to SMD infection is under the control of independent nonallelic genes [19]. Explained the inheritance of sterility mosaic assuming the presence of four alleles at two loci [14]. Two alleles control resistance, one of which was dominant and the other recessive to tolerance. The allele responsible for susceptibility was found dominant over the other three alleles; *albl* susceptible; *a3b3* tolerant; *a2b2* and *a4b4* resistant. Reported SMD was governed by four independent nonallelic genes [20]. The presence of at least one dominant and one recessive gene was necessary for resistance. Also reported four independent nonallelic genes (*Sv1*, *Sv2*, *Sv3* and *Sv4* controlling sterility mosaic disease [20]. At least one dominant and one recessive gene were necessary to express resistance. Resistance was controlled by four independent loci, two duplicate dominant genes (Sv1 and *Sv2*), and two duplicate recessive genes (sv1 and sv4.). For expressing resistance reaction at least one dominant allele at locus 1 or 2 and homozygous recessive at locus 3 or 4 are necessary. Identified random amplified polymorphic DNA (RAPD) [21] primers and developed a sequence characterized amplified region (SCAR) marker associated with pigeon pea sterility mosaic disease (PPSMD) resistance in pigeonpea cross ICPL-7035 x ICPL-8863. Random marker OPA18800 only revealed polymorphism in resistant and susceptible lines, indicating that the marker OPA18 was associated with PPSMD resistance in ICPL-7035. End sequences of these markers were used to design allele-specific sequence characterized amplified region (SCAR) marker SCAR 816(16f/r), which was present in all generations (parents, F_1 and F_2), to identify the transfer of the SMD resistance gene to susceptible lines [22]. Show that SMD resistance was under the control of one recessive gene [23]. They used AFLP markers to screened two parental genotypes for identification of polymorphic AFLP markers. Identify four AFLP markers E-CAA/M-$GTG_{150.}$ E-CAA/M-$GTG_{60,}$ E-CAG/M-GCC_{150} and E-CAG/M-$GCC_{12\ 0}$ found associated with SMD resistance can be used for MAS [23]. Also found SMD resistance governed by one recessive gene [24].

Relation between SMD Resistance and Mite Interaction: SMD resistance in some genotypes is due to immunity to PPSMV, in others to resistance to *Aceria cajani,* and in a few others to resistance to both organisms [25]. With regard to mite resistance, it is known that some SMD-resistant genotypes have a thicker leaf cuticle and epidermal cell wall than those of mite-susceptible genotypes [26]. Conceivably, the thick cuticle prevents the short mite stylets reaching epidermal cells, preventing feeding altogether [27]. A complicating factor in determining the precise nature of the resistance mechanism is our finding that the reproduction of *A.cajani* is much greater on PPSMV-infected plants than on healthy plants of the same genotype, confirming some earlier field observations [28]. There seems therefore to be a beneficial relationship between the vector mite and the virus it transmits, and this may explain why mites are rarely found on PPSMV-resistant pigeonpea genotypes.

Molecular Markers and Phytophthora and Alternaria Blight: A few research papers describing the genetics of Phytophthora and Alternaria blight resistance are available. But use of molecular markers to study resistance in pigeonpea against Phytophthora and Alternaria blight is in primitive stage only. Resistance to Phytophthora blight was reported to be governed by a single recessive *(Pdl) gene* [29]. Alternaria blight was controlled by a single recessive *(abrl)*

gene [[30]; further [31] confirmed that it is governed by monogeneic recessive gene. Found that transfer of PSB resistance to virulent race P3, from wild species of pigeonpea (*cajanus platycarpus*) to cultivated pigeonpea cultivars by means of embryo rescue method [32]. The nature of resistance found to be monogenic and recessive.

Genetic Transformation of Pigeonpea: The introduction of specific genes into pigeonpea to improve pest and disease resistance and also to improve nutritional quality could be achieved by genetic engineering or genetic transformation approaches [33]. Genetic transformation technology relies on the technical aspects of plant tissue culture and molecular biology to develop commercial products. The major components for the development of transgenic plants are: (i) the development of reliable tissue culture regeneration systems, (ii) preparation of gene constructs for transformation with suitable vectors, (iii) efficient techniques of transformation for the introduction of genes into the crop plants, (iv) recovery and multiplication of transgenic plants, (v) molecular and genetic characterization of transgenic plants for stable and efficient gene expression, (vi) transfer of genes to elite cultivars by conventional breeding methods if required, and (vii) evaluation of transgenic plants for their effectiveness in providing the desired characteristic and general field performance [34]. For commercialization of transgenic crops, additional aspects must be addressed, including (i) biosafety assessment including food, feed and environmental safety, (ii) intellectual property rights and (iii) consumer acceptance [35, 36]. However, in the catalogue of abiotic stresses an extensive need for the development of transgenics was felt for resistance to drought, water-logging, salinity and thermo-insensitivity. While the importance of transgenic for pigeonpea improvement was widely accepted, it is important to consider that there is no reproducible protocol for genetic transformation of pigeonpea [37]. It thus emerged that substantial efforts have to be put in this direction to make use of transgenic technologies for pigeonpea improvement. A few reports on the development of genetically engineered disease or pest resistant pigeonpea plants are summarized in this section. One of the many natural defense mechanisms plants use to resist pathogen attack is to accumulate proteins (e.g. chitinases) active against disease-causing organisms. In some cases, where this mechanism is too weak or appears too late to fully protect the plant, engineering constitutive expression of a defense protein can boost tolerance to fungal pathogens [37]. Transgenic pigeon pea plants, expressing a cowpea protease inhibitor gene or a protective antigen of the Rinderpest virus, have been obtained using *Agrobacterium* -mediated gene transfer or bombardment with micro-particles [38]. Transgenic plants with resistance to major biotic constraints are being developed and tested by ICRISAT and its research partners, especially for legume crops [39]. For example, they are investigating, in collaboration with scientists from Scotland (UK), the enhancement of resistance to *Botrytis* gray mold of chickpea using polygalacturinase inhibiting protein (PGIP) genes. Achieved transformation of pigeon pea (*Cajanus cajan* L. Millsp.) [40] using *Agrobacterium tumefaciens* strain GV2260, containing the construct of isolated cowpea protease inhibitor gene (*pCPI*; Accession no.: AJ271752). The gene was driven by CaMV 35S promoter containing kanamycin resistance as plant selection marker. This is a step forward in developing transgenic pigeon pea resistant to chewing insects, mainly pod borers. Report the development of transgenic pigeonpea with resistance to fungal disease, by the transfer of a rice chitinase gene to pigeonpea [41]. The rice chitinase gene harboured in the plasmid pCAMBIA 1302: RChit was delivered via the Agrobacterium-mediated method to the cotyledonary node explants followed by subsequent regeneration of complete plants on selection media containing hygromycin. Putative transformed pigeonpea plants were recovered with stringent selection pressure and confirmed using molecular techniques. Stable integration and expression of the chitinase gene has been confirmed in the T0 and T1 transgenics through molecular analysis.

Gaps in Research of Pigeonpea Disease Resistance and Possible Lines of Research in the Future: The genetics of disease resistance and development of resistant varieties in pigeonpea is suffering from little concerted research effort. Although, several programs for improvement of agronomical traits in pigeonpea utilizing both conventional and molecular methods are ongoing, but the genetic basis of resistance against most of the diseases in pigeonpea is not known to date. Knowledge of genetics of disease resistance in pigeonpea will be very helpful for the development of resistant varieties. There is a need to complement existing

conventional methods with molecular methods by changing focus from phenotypic to genotypic breeding and use of molecular markers. Use of molecular markers in diverse mapping populations in pigeonpea will facilitate the construction of a genetic map, mapping, and map based cloning of disease resistance genes, quantitative trait loci (QTL) mapping, and the integration of phenotypic data across the different mapping populations. Furthermore, the development of molecular markers will be helpful in pigeonpea improvement via marker assisted selection (MAS) or transgenic approaches.

In summary, there is need to improve disease resistance in pigeonpea cultivars. Molecular tools promise to facilitate improvement efforts by providing information at molecular level. The complementation of these tools with conventional methods is needed for optimum results.

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