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Progresses in Biotechnological Applications to Coffee Wilt Disease

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Abstract

Molecular markers and biochemical compositions are vital to distinguish genotypes for specific traits and rapid means of identification of important traits. Random amplification of polymorphic DNA- polymerase chain reaction (RAPD-PCR) analysis, single-enzyme, agarose gel-based amplified fragment length polymorphism (AFLP), enzymatic digestion of the intergenic spacer (IGS) region, and inter-simple sequence repeat (ISSR) analysis and amplification result showed that there exist distinguishable Arabica and Robusta coffee wilt disease (CWD) pathogenic forms. Based on the translation elongation factor, the calmodulin gene, and the histone gene analysis; four phylogenetic species (PS) within the *Gibberella xylarioides* complex were identified and are consistent with the four biological species (BS) groups (BS1, BS2, BS3, and SG4). A single mating gene (MAT) gene of two different forms (alleles) (MAT1-1 and MAT1-2) has been described for the Fusarium genome. Polyclonal antibodies rose against the endo-antigens from *Fusarium xylarioides* of 27 kDa, which is a promising tool for the rapid, sensitive, and accurate detection of the pathogen in soil and plant parts. QTL mapping studies should be initiated using the double haploid CWD-susceptible/ resistant parents and their progenies to identify molecular markers and/or quantitative trait loci associated with resistance to assist in breeding resistant varieties development and isolation of resistance genes for creating bacterial artificial chromosome libraries. However, applications of different biotechnological and biochemical techniques are found in infant stage to CWD studies and to provide a rapid solution to the area. Therefore, further efficient and reliable disease screening methods are required, such as in-vitro screening. Moreover, studies should be conducted to discover genetic markers for CWD resistance in the coffee genome and QTL mapping study to marker-assisted selection and develop CWD resistant varieties.

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Arabica coffee, Coffee wilt disease, Molecular markers.

Introduction

Morphological variation only does not reflect true genetic variation that exists at the DNA level because morphological markers are confounded by environmental, pleiotropic and epistatic effects. Besides, assessment of genetic diversity using morphological traits in perennial plants, such as coffee, often requires a lengthy and expensive evaluation during the entire vegetative growth period (Weising *et al.*, 2005). Detecting and quantifying genetic variation in crop species is important for improvement of desirable characteristics in a breeding program. Lashermes *et al.*, (1996) reported the importance of DNA-based markers in accurately detecting genetic factors in coffee. Various molecular markers, such as restriction fragment length

polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR), simple sequence repeats (SSR), and expressed sequence tag derived simple sequence repeats (EST-SSR) have been used in coffee genetic diversity studies (Steiger, 2002). More recently, a new type of molecular marker known as a sequence related amplified polymorphism (SRAP) markers were also successfully used to discriminate between parents in hybrid identification and therefore has great potential in coffee breeding programs. In addition to the above, single-nucleotide polymorphisms (SNPs) and PCR-RFLP markers were used in coffee genome analysis (Mishra *et al.*, 2011).

Coffee resistance improvement to different diseases have been mainly focused on finding new sources of resistance and a better understanding of both the pathogens diversity and the host defense mechanisms. In view of the complexity of plant disease resistance to pathogens, which is becoming increasingly apparent as newtools in genetics, cell biology, biochemistry and molecular biology are being used. According to Bieysse (2007) and Rutherford (2005), single-enzyme, agarose gel-based amplified fragment length polymorphism (AFLP) analysis and amplification and enzymatic digestion of the intergenic spacer (IGS) region, showed that two distinguishable pathogen populations exist, those collected from Arabica and those from Robusta. Rutherford (2006) in his inter-simple sequence repeat (ISSR) analysis of *Fusarium xylarioides* isolates from CWD infected Robusta or Arabica coffee observed that these plants were affected by distinct strains. In addition, a study using infection tests in Ethiopia showed host-strain specificity where isolates from Arabica coffee plants were pathogenic only to seedlings of Arabica coffee (Girma *et al.*, 2005). The pathogen survives in the soil and difficult to control by fungicides. However, it may be controlled by antagonistic biological control agent such as Trichoderma species, which has been reported to control the pathogen by up to 71% in-vitro (Alemu, 2012).

Molecular and serology techniques of plant disease detection have been well-established (Sindhuja *et al.*, 2010). Early detection of CWD pathogen in the host when the infection levels are very low makes the test simple and rapid, in plant proliferation of pathogen, determination of fungal biomass in infected tissues, characterization of genetic races of the pathogen (Bhuvanendra *et al.*, 2010), based on their protein banding pattern and immuno-reactivity patterns. Studies had been initiated at CORI to characterize CWD resistance using molecular markers. Mapping studies also initiated using the double haploid CWD-susceptible/ resistant parents and their progenies to identify molecular markers and quantitative trait loci (QTL) associated with CWD resistance to assist breeding resistant varieties and isolate resistant genes for creating bacterial artificial chromosome libraries. Use of double haploids shall minimize the effect of heterozygosity (Phiri and Baker, 2009).

The objective of this review is to realize the application of different biotechnological tools to characterize and variation study of the pathogen (*Gibberella xylarioides*) and the status of molecular technique advancement to identify molecular markers to develop CWD resistance.

Molecular approaches to coffee diseases

Efficient and reliable disease screening methods are required for a successful variety development program. Molecular markers linked to resistance provide the potential to screen resistance in a large population of plants at any stage of plant development. Where several genes confer resistance, markers have the advantage over morphological assessments, because plants carrying multiple resistances (broad-based resistance) can easily be differentiated from those carrying a single gene (narrow-based resistance). Attempts have been made to screen for DNA markers linked to CBD resistance in Catimor and Rume Sudan coffee varieties (Gichuru *et al.*, 2008). They concluded that the gene conferring the resistance was localized within an 11 cM segment and the locus carries a major resistance gene designated as *Ck*-1. In the case of coffee leaf rust the genetic analysis of an $F_{2\ \text{population}}$ consisting of 224 plants derived from a crossing of Híbrido de Timor UFV 427-15 (resistant) with CatuaíAmarelo IAC 30 (susceptible) showed that a dominant gene confers the resistance of coffee to race II of Hemileiavastatrix(Diola *et al.*, 2011). From a genetic map saturated with 25 amplified fragment length polymorphism (AFLP) markers linked to the resistance gene, they developed a high-density genetic map with six sequence-characterized amplified region (SCAR) markers delimiting a chromosomal region of 9.45 cM and flanking the dominant gene at 0.7 and 0.9 cM.

Biochemical studies showed that a significant increase in proteolytic enzymes and in peroxidase activity in coffee varieties after inoculation with *C. kahawae* (Gichuru, 1993; Gichuru *et al.*, 1997). Peroxidase activity of extracts obtained from non-inoculated hypocotyls, and

detached and attached berries did not reveal differences between the resistant and susceptible varieties.But, the highest increases in enzyme activity were associated with susceptibility rather than with resistance (Gichuru *et al.*, 1997).

Molecular genetic diversity for coffee wilt disease pathogen

There are three basic ways to distinguish between different individual fungi (accessions) collected in the field (Phiri and Baker, 2009). These are morphological (how they look to the naked eye or under the microscope), biological (if the different individuals interbreed or not), and molecular (one or more molecular tests to determine the degree of similarity in the molecular structure, either of a gene, or the DNA between genes).

Girma (2004) and Lepoint (2006) conclude that only the Arabica and Robusta strains can be distinguished visually in the laboratory by growing the strains from each on a rich agar potato dextrose agar (PDA) medium.

An orange pigmentation is apparent on young CWD cultures from Robusta grown on a PDA or cereal agar (CA) medium, which is absent in isolates from Arabica. But beyond this gross level, no visual separation is possible.

One way to resolve how closely strains are related is to mate them, if the sexual form of the disease is available, which is the case for CWD (*Gibberella xylarioides*), which is heterothallic, meaning that there are two forms present, one with the MAT-1 version of the mating gene and the other with the MAT-2 version (Girma,2007). If they produce a viable offspring, it is reasonable to lump them under the same species heading, since this is the principal definition of a species. If they do not cross or they produce sterile offspring, it is reasonable to suggest that they are different species.

Lepoint (2006) carried out a series of crossings of different strains in the laboratory. Reproductive success was scored on a scale of 0 to 4: completely sterile (0), proto-perithecia only (1), barren perithecia (2), perithecia containing unidentified structures (3) and fully fertile (4). From the result he distinguishes three species by biological species recognition (BSR) that correspond closely to Arabica, Robusta and Excelsa strains, as well as a residual group of incompatible strains which are lumped as a further species, making four in all.

Molecular methods offer the greatest possibilities for differentiation between strains. The main problem is to find parts of the genome to analyze that might correspond to meaningful divisions, i.e., not too coarse-grained that little differentiation is seen, but not so fine-grained that every strain collected is deemed unique.

The story of molecular studies of CWD is one of gradually increasing resolution of what initially looked like an almost identical collection of strains. The most meaningful distinguishing markers are those that relate closely to the ability of putative species to interbreed, but finding such reliable markers can be very difficult (Phiri and Baker, 2009).

Random amplified polymorphic DNA (RAPD) analysis

RAPD was one of the first molecular techniques used to look for differences between organisms. The technique analyses randomly chosen parts of the genome for differences between genomes of two or more organisms, and can successfully distinguish between species but is now often regarded as to unreliable when it comes to determining between strains of the same species.

Twelve 10–16 oligonucleotide primers, that indicating inter- and intra-species variation in the genus *Fusarium* (Hering, 1997), were selected and used. Out of 12 oligo-nucleotide primers, RAPD-PCR analysis with five oligo-nucleotide primers produced clear informative and reproducible polymorphic DNA banding patterns (Girma *et al.*, 2005). The analysis showed that all the recent Arabica isolates including those isolates derived from the same ascus had monomorphic RAPD amplifications and clustered into a single group indicating homogeneity of the population. There were, however, clear DNA polymorphism among *Gibberella xylarioides* trains from *Coffeaarabica, C. canephorae* and *C. excels* with varying fragment lengths. *Gibberella xylarioides* was distinctly polymorphic to *Fusarium stilboides, F. solani, F.* cf. *eumartii* and *F. lateritium* var. *Longum* (Figure 1) conforming to the taxonomic classification of these species. Even though they originated from diverse environments like host cultivar, agro ecological zones as well as production systems and varied significantly in aggressiveness in the pathogenicity test. The historic Arabica strain of 1971, however, seems to be slightly different from the recent Arabica collections that may implicate little genetic changes in the pathogen populations over the last 30 years (1971–2001). The results of RAPD-PCR markers corroborated the

existence of host specialization into at least two pathogenic forms within *Gibberella xylarioides* populations. These are *Gibberella xylarioides* f. sp. *abyssiniae*(anamorph: *Fusarium xylarioides* f. sp. *abyssiniae*) for the fungal strains attacking only *C. Arabica* and *Gibberella xylarioides* f. sp. *canephorae*(anamorph: *F. xylarioides* f. sp. *canephorae*) pathogenic to *C. canephorae* and *C. excels.*

According to Bieysse (2007) at CIRAD and Rutherford (2005) at CAB International, single-enzyme, agarose gel-based amplified fragment length polymorphism (AFLP) analysis and amplification and enzymatic digestion of the intergenic spacer (IGS) region, generally revealed little genetic variation among the CWD isolates. But itshowed that two distinguishable pathogen populations exist; those collected from Arabica and those from Robusta in Uganda, DRC and Tanzania. These findings were confirmed by a polymerase chain reaction (PCR) analysis of ISSR DNA sequence.

Individual gene studies

The subjectivity of determining potential species boundaries within the *Gibberella xylarioides* complex (GxC) can be reduced by the analysis of several genes. Lepoint chose three different genes to look for differences: the translation elongation factor 1-a (tef gene), the calamodulin gene and the histone gene. These are all so-called nuclear house-keeping genes that are permanently switched on and are vital for the correct functioning of the cell, and hence differences found are much less likely to be due to the random genetic variation and much more likely to represent significant functional differences. By analyzing, these three genes in different strains and using a statistical technique called maximum parsimony analysis with the help of a phylo-genetics software package (Swofford, 2001) and Lepoint (2006) found the following differences:

Translation elongation factor *tef 1-a*: Based on tef analysis, three groupings (clades) were identified, corresponding to (i) BS3, (ii) SG4 and (iii) a third group containing both BS1and BS2.

Calmodulin CL: Based on a 609 bpcalmodulin fragment analysis, *Gibberella xylarioides* complex *(*GxC) divides into two clades, clade 1 contains BS1 strains while clade 2 contains BS2, BS3 and SG4 strains.

Histone 3 (H3): Phylogenetic analysis of the 459 bp H3 sequence revealed six single nucleotide polymorphisms (SNPs). Three clades could be distinguished within GxC, clade 1 containing BS2 strains, clade 2 including all SG4 strains while clade 3 containing BS1 and BS3 strains. Combining the three: Using all the above, the maximum parsimony analysis suggests that four phylogenetic species (PS) are recognizable within GxC and are consistent with the four BS groups, with a further subdivision so that BS1 and BS2 form a sister clade, whereas BS3 and SG4 form another. With additional analysis of strains from GiC, it was also confirmed that GxC and GiC (the *G. indica*complex that contains pigeon pea wilt *F. udum*) are closely related sister clades.

The mating gene (MAT)

Study differences in the mating gene (MAT) that is known to control the fungal sexual reproduction is also possible means of distinguishing between species of fungi. It is likely that differences in the MAT structure between strains are related to their ability to cross and hence is an interesting place to look for taxonomic markers. Indeed, previous work on a non-Fusarium species has shown that a single mutation in MAT can greatly reduce sexual compatibility (Ferreira *et al.*, 1998). A single MAT gene of two different forms (alleles), MAT1-1 and MAT1-2, has been described for the Fusarium genome (Coppin *et al.*, 1997; Kronstad and Staben, 1997). Each individual of heterothallic species such as GxC has either the MAT1-1 or MAT1-2 version of the gene. The MAT1-1 gene consists of three subsections or open-reading frames: MAT1-1-1, MAT1-1-2 and MAT1-1-3, whereas the MAT1-2 gene has only one frame called MAT1-2-1.

Lepoint (2006) looked at the structure of these genes in different strains of GxC to find out if differences might correspond to those found from other taxonomic methods. The result shows a phylogenetic species (PS1) was identified, containing all the Ethiopian Arabicastrains.

This could be further subdivided into two groups based on differences in MAT1-1-1 and MAT1-1-3 frames. Robusta associated strains from Uganda and DRC from the recent upsurge formed a second PS2 group. PS3 includes only two strains, a CAR strain from Excelsa coffee from the 1950s (BBA 62457) and a strain of unknown provenance collected in 1964. A further species group, PS4, contains strains from CAR, Côte d'Ivoire and Guinea all isolated in the 1950s and 1960s, but the designation of this group is less certain, because of conflicting tendencies from analyses of MAT1-2,

MAT1-1-2, MAT1-1-1 and MAT1-1-3. This area needs further analysis with more material from these countries, if it could be found.

Immunodiagnostic potential of a 27 kDa protein of *Fusarium xylarioides*

In recent years, molecular and serology techniques of plant disease detection have been well established (Sindhuja *et al.*, 2010). The molecular methods, which are usually PCR based is where a specific base sequence of the genetic material of the pathogen is utilized, while in serological or immunological methods, the microbial protein (antigen) associated with a pathogen is introduced into an animal that produces specific antibodies against the antigen. The techniques are gaining importance due to their specificity, sensitivity and rapidity. Such advanced plant disease detection techniques can provide rapid, accurate, and reliable detection of plant diseases in early stages for economic, production, and agricultural benefits (Sindhuja *et al.*, 2010). In this review identification of the F. xylarioides immunodiagnostic target protein that has produced specific polyclonal antibody in rabbit. The specific antibodies can be packaged into an immunodiagnostic procedure and integrated with the current culture based-techniques for better detection of the pathogen both in soil and coffee plant parts. Early detection of CWD pathogen in the host when the infection levels are very low makes the test simple and rapid, in-planta proliferation of pathogen, determination of fungal biomass in infected tissues, characterization of genetic races of the pathogen, based on their protein banding pattern and immuno-reactivity patterns (Bhuvanendra *et al.*, 2010).

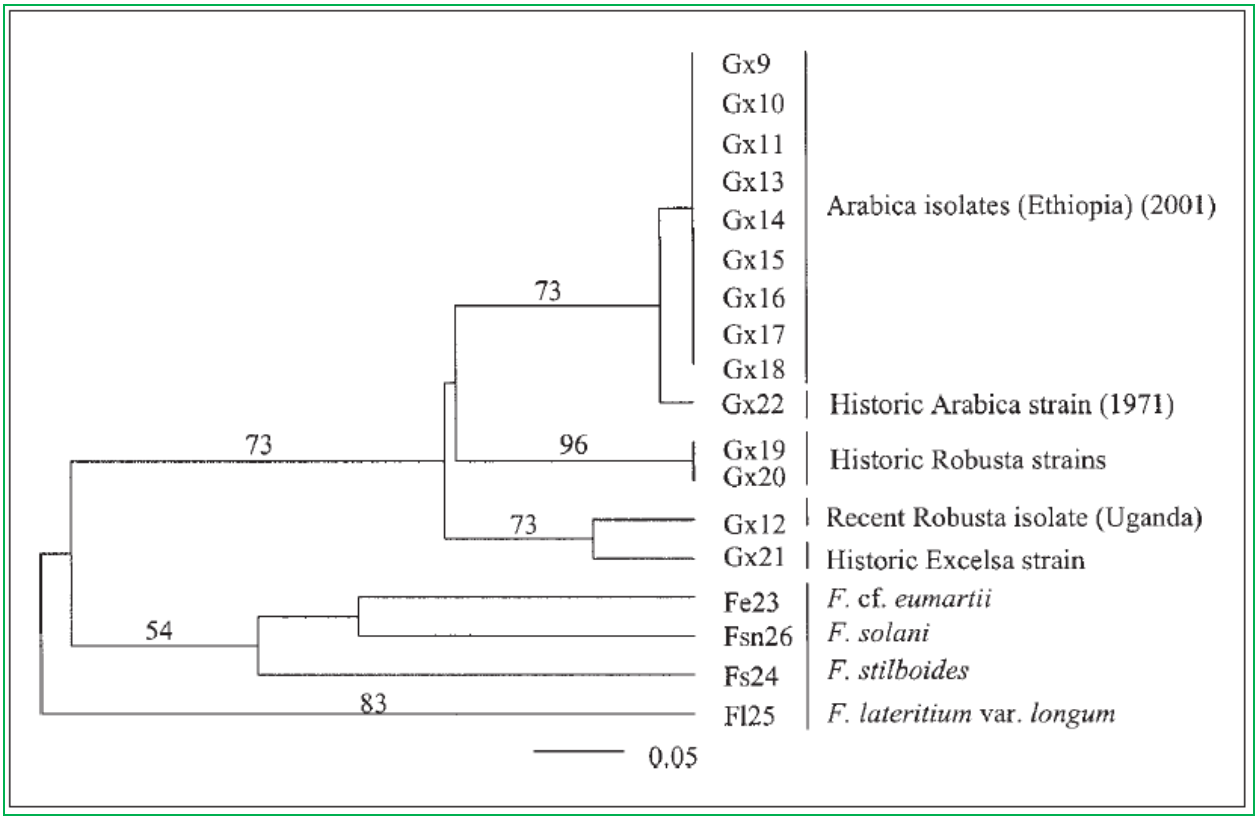
Marker assisted selection for disease resistance in coffee

Molecular markers have been used in coffee for introgression assessment, determination of mode of inheritance of disease and pest resistance, assessment of beverage quality, and analysis of quantitative loci (QTL), all of which have great implication for future breeding. Efficient use of genetic variation in wild species involves the genetic determination of the desirable traits and the ability to introgress the desirable DNA segments from wild species to the cultivated species (Prakash *et al.*, 2004) without affecting quality traits (Herrera *et al.*, 2002). Markers linked to specific traits such as resistance to leaf rust (De Brito *et al.*, 2010; Diola *et al.*, 2011; Herrera *et al.*, 2009; Mahe *et al.*, 2008; Prakash *et al.*, 2004 and 2011), coffee berry disease (Agwanda *et al.*, 1997; Gichuru *et al.*, 2008), and root rot nematode (Noir *et al.*, 2003) have been identified. Prakash *et al.*, 2011 have successfully applied Marker assisted selection (MAS) in achieving durable rust resistance. They were able to distinguish the presence or absence of the S_H3 gene using the arabica cultivar S.795, a cultivar derived from S.26, a spontaneous hybrid of *Coffea arabica* and *Coffea liberica* using two SCAR markers closely linked to the S_H3 gene (Sat244 and BA-124-12K-f). The marker Sat244 was more efficient in distinguishing the homozygous and heterozygous status of the S_H3 gene.

Agwanda *et al.*, (1997) performed studies to identify RAPD markers associated with CBD resistance using SL28 and Caturra as susceptible cultivars and Rume Sudan, K7, and Catimor as resistant donors. Three RAPD markers were found to be closely associated with resistance to CBD in Arabica coffee, controlled by the *T* gene found in the varieties Hibrido de Timor and Catimor. This provides an efficient way to select for the *T* gene in crosses involving Catimor and Hibrido de Timor through MAS. In the Studies of molecular markers identification for CBD resistance, eight AFLP and two microsatellite markers were tightly linked to resistant phenotypes, which were mapped to one unique chromosomal fragment introgressed from *C.canephorae* (Gichuru *et al.*, 2008). The development of DNA markers linked to CBD resistance genes would considerably improve the efficiency of breeding programs by allowing selection at an early stage and gene stacking to increase the chances of high levels of durable resistance (Lashermes *et al.*, 2000b; Gichuru *et al.*, 2008).

In conventional breeding programs, incorporation of CWD resistance genes into commercial clones that should also have good quality traits is likely to take a very long time. Molecular techniques should be adopted to facilitate the breeding and selection process. Studies had been initiated at CORI to characterize CWD resistance using molecular markers.

Fig.1 Clustering of *Gibberella xylarioides* strains from *Coffea arabica, C. canephorae, C. excels* and other *Fusarium* species (Girma *et al.*, 2005)



Mapping studies could be initiated using the double haploid CWD-susceptible/ resistant parents and their progenies to identify molecular markers and or quantitative trait loci (QTL) associated with CWD resistance to assist in breeding resistant varieties and isolation of resistance genes for creating bacterial artificial chromosome libraries and it found under progress. Use of double haploids shall minimize the effect of heterozygosity (Phiri and Baker, 2009). In Ethiopia, MAS and QTL mapping for CWD resistanceis not started yet. However, it is highly significant to solve the CWD problem in the country and will be functional in the near future.

There are three basic ways to distinguish between different individual fungi (accessions) collected in the field. These are morphological, biological, and molecular methods. Arabica and Robusta strains can be distinguished visually in the laboratory by growing the strains from each on a rich agar potato dextrose agar (PDA) medium. RAPD-PCR, single-enzyme, AFLP, IGS region and ISSR analyses showed that two distinguishable pathogen populations exist in CWD; Arabica (responsible for Arabica coffee in Ethiopia) and Robusta (responsible for Robusta coffee in Uganda, Tanzania, and DRC) pathotype.

By analysis of three different genes (the translation elongation factor, the calamodulin gene and the histone gene), four phylogenetic species (PS) within GxC were identified and are consistent with the four BS groups (BS1, BS2, BS3 and SG4). A single MAT gene of two different forms (alleles), MAT1-1 and MAT1-2, has been described for the *Fusarium* genome. Studies showed that 27 kDa *F. xylarioides* protein is species specific to CWD pathogen and development of monoclonal antibodies against the 27 kDa protein would enhance specificity, homogeneity and production of the antibodies.

Double haploid CWD-susceptible/ resistant parents and their progenies study is initiated to identify molecular markers and develop quantitative trait loci (QTL) associated with CWD resistance to assist in resistant varieties selection based on the target markers at CORI, Uganda. This study is paramount for other African countries, particularly for Ethiopia to see and start this

type of works and develop CWD resistant varieties in short time, because Ethiopia is endowed a full of coffee genetic resources, diversity and source of gene pool for many disease problems.

The Way forward

In the future, there should be conduct more investigation to discover genetic or DNA molecular markers in the coffee genome linked to CWD resistance for easily screening of large population of genotypes at any stage of plant development, and mapping study. Efficient, rapid and reliable screening methods should be developed to detect the presence of CWD at early time or stage, and a test could be also distinguishing it from other *Fusarium* species. Biochemical analysis for different CWD reaction groups should be done in order to generate which chemical composition and constitution is important and a contribution for resistance. Generally, the biotechnological applications to CWD are found at in fate stage, and more research should be done using molecular techniques in order to develop CWD resistant varieties.

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