



ROLE OF CRISPR/Cas IN CONTROLLING BIOTIC STRESS IN BLACKPEPPER

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ABSTRACT

Black pepper (*Piper nigrum* L.), the king of spices is one of the most important traditional spices cultivated all over world. Over the past 15 years, there has been a noticeable decline in crop production and area due to biotic and abiotic stress. Despite the efforts made to develop and select a number of black pepper varieties with high yield potential and disease tolerance, the situation has not improved in a decade. Quick wilt caused by *Phytophthora capsici*, one of the major soil-borne fungi can destroy black pepper crops and cause heavy loss in the plantations. All plant parts are vulnerable to infection, which results in significant decrease in gene expression, thereby inducing heavy mortality rate. Different resistant varieties are raised based on different breeding programs to control the disease and helps in maintaining black pepper production. Such labor-intensive, unfocused breeding initiatives that take so much time and effort cannot keep up with the needs for higher crop production. Currently, a novel gene editing technique known as the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas technology, has succeeded in enhancing crop quality that increase yield, quality as well as to improve resistance to biotic and abiotic stresses. The main objective of this review is to identify the role of CRISPR/Cas technology in controlling the quick wilt fungi by genome editing. Recent improvements in CRISPR/Cas genome editing allow for effective targeted modification in the majority of crops, which promises to hasten crop development, especially in commercially important crop like black pepper.

Keywords: Black Pepper, Quick wilt, CRISPR/Cas, Genome editing.

1. INTRODUCTION

Black pepper [*Piper nigrum* (L.)] the king of spices is a perennial vine belonging to the family *Piperaceae* [1]. The name pepper is derived from the Sanskrit word pippali meaning “long pepper” [2]. Black pepper is extensively used as spice and in medicine [3]. Pepper is an ancient and traditional crop native to South Asia and South East Asia. Out of major countries in the world, India is one of the major producer, consumer and exporter of black pepper, particularly in the state of Kerala [4]. The black pepper plant is a perennial woody vine by means of its aerial roots it is growing up to 10 meters of height [5]. Its green color leaf pattern is in alternate arrangement and flowers that grow in clusters have opposite spikes. The fruits, which are small, round, berry-like sometimes called as peppercorn or drupes about 5 mm or 0.2 inch in diameter. At maturity, it becomes yellowish red with a single seed. Seeds have a pungent and penetrating smell with a hot taste. The chemical piperine is the reason for its characteristic flavor [6] and seeds also contain chemicals such as chavicine, piperidine, and piperettine

[7]. The plant requires an ample rainfall combined with high temperature and cooling for best possible conditions for the growth of plant [8]. It takes about 2 to 5 years to start bearing fruit and they can continue to produce for up to 40 years. This crop grow in a temperature ranges between 10 and 40 degree Celsius with a pH of soil 5.5 to 6.5. Pepper appears as different colors based on their ripeness, harvesting, processing. Green, black and white are the different colors. Black peppercorn is obtained by when fruit turn into red. After that it will immersed in hot water for about 10 minutes, which will turn into dark brown or black color in an hour. Then they are kept under sunlight for three or four days until they become dried, wrinkled and black. Green peppers are picked before they fully mature. White peppercorns are yielded when the red peppers are soaked and peeled. In Kerala, Wayanad and Idukki districts are the largest producers of pepper. But hundreds of acres of pepper now are in the threats of quick wilt disease. Many breeding methods are adopted to control the disease. But still are in the verge of threat [9]. The main Wayanadan pepper varieties are

'Sugantha kurumulaku', 'Karikonta', 'Cheruvally', 'Kalluvally', 'Uthirankotta' 'Balankotta', 'Karimunda' [10].

2. QUICK WILT

Black pepper is affected by various pathogens [11]. Of all these, quick wilt disease is the most damaging one [12]. Studies reported that quick wilt is a severe disease in different pepper growing countries. Since 1902, it was first observed in Wayanad region and found that it is affected by *Phytophthora capsici* and reported first in 1966 [13].

3. CAUSATIVE ORGANISM

Quick wilt of black pepper is caused by *Phytophthora capsici*. It was first reported on chili pepper (*Capsicum frutescens*) by Leonian from New Mexico in 1922 [14]. It is an oomycete plant pathogen also known as a water mold. It produces zoospores which can swim through waterfilms and wet soil to invade new host plants. The optimum temperature for the growth of fungus is 25-30 degree Celsius. There are asexual and sexual reproductive structures. Asexual structures consist of sporangiophores, sporangia, chlamydospores and hyphae. Sexual structures are antheridia, oogonia and oospores. *Phytophthora palmivora* (Butler), the disease's original causative agent, has been replaced with *Phytophthora capsici* [15]. Mycelium is hyaline, branching, and non-septate, however elderly hyphae may include a few septa. Typically, hyphal branches originate at right angles and are frequently abnormally bloated, tuberous, and in diameter. The sporangia are hyaline, ovoid to pyriform or occasionally round to lemon shaped, non-pedicillate, and have a predominating, hemispherical papilla at the apex [16]. Sporangia are infrequently formed or practically nonexistent in culture. The zoospores range in shape from reniform to oval, are biflagellate, and are motile for 20–30 minutes before losing their flagella, becoming encysted, and then germinating through germ tubes. Oospores are produced in large quantities by both aerial and submerged mycelia in the medium. Hyaline, spherical to circular oogonia are present. Persistent amphigynous antheridium surrounds the oogonial stalk. The oospores can range in shape from circular to spherical, and they can either germinate through germ tubes or by the breaching of oosporic walls [17].

4. SYMPTOMS

The pathogen infects all parts of black pepper like leaves, stems, spikes, collar and root are prone to infection.

4.1. Soil phase

The fungus starts infection from root to collar or foot of the plant so called as foot rot or collar rot. Within two to three weeks, the vine starts to rot and die because the stem close to the ground becomes infected. The affected area has a foul smell. The subterranean stem and the root system are both affected as the necrosis spreads downward. Collar infection is destructive and also affects through runner shoots. At the month May-June, infection starts because of high moisture and optimum temperature which facilitates efficient growth of fungus. In early days of infection, foliar yellowing is the major symptom. As the vine matures, its leaves fall off, its aerial branches split at the nodes, and eventually vine dies. Sometimes the vine eventually dies without exhibiting any foliar yellowing. Through association with soil, water, and roots, the disease propagates.

4.2. Aerial phase

Die back is a major symptom affecting the aerial branches. Discoloration occurs at the site of infection of branch and leaves, tender and woody stems with dark wet spots increase in size and affects major portion of leaf. Sometimes, a pale whitish color is noticed in middle of spot. The fungus also affects leaves, resulting in dark brown patches that quickly spread to cover a considerable area of the leaf. The lesion exhibits typical fimbriate borders. On rare occasions, a concentric zone of the spots with a light whitish centre is seen as well. Foliar infection causes variable degrees of defoliation because infected leaves drop off. When an infection spreads to a branch, the infected branch rots and turns a dark brown color. Branches that have been infected further show foliar yellowing, defoliation, and wilting. Additionally, fungus can infect spikes at any time, causing rotting and the eventual fall of the infected spikes. Foliar infection happens during June and July, when there is a lot of rain. Rain splashes allow disease to travel from lower to upper portions of the vegetation.

4.3. Slow wilt vs. Quick wilt

Variable levels of foliar yellowing and defoliation are brought on by slow decline infection. Nowadays slow wilt is called as slow decline. *Radopholus similis* and *Meloidogyne incognita*, two significant soil-borne plant parasitic nematodes, may infect feeder roots, resulting in the devastating disease complex. This connection changes from location to place. In diseased plant detritus, fungus can survive. Infected plant waste, soil, collateral, and other hosts like Solonaceous, Malvaceous, and Leguminaceous plants serve as sources of inoculums.

These are cysts and egg masses. Juveniles in their second stage that are autonomous and may be spread by water. Disease development is favored by wet weather, light and loamy soils. In quick wilt addition to soil, fungus can live in diseased plant detritus. These vines may bounce back following the rains and continue to grow for more than two seasons before the root infection results in collar rot and the vine's death. The onset of sickness is favored by the rainy season from October to November.

4.4. Root rot

The infection weakens the root system causes different degrees of root rotting and leaves lead to foliar yellowing from January onwards with gradual depletion of soil moisture [18]. The vines with severe root infections dry up during this period. The vigor and yield of affected vines steadily deteriorate. Intense foliage yellowing with slow soil moisture loss is present on the diseased vines with a degenerating root system starting in January. During this time, the severely infected vines shrivel up [19].

5. EPIDEMIOLOGY AND DISEASE CYCLE

The main source of inoculum appears to be dried vines in the gardens and plant debris from diseased plants. Since *P.capsici* is a pathogen that thrives in wet conditions, its activity is influenced by moisture regimes in both the soil and the vine's aerial parts. The advent of the south-west monsoon in May or June marks the beginning of the monsoon season, which lasts through August and then continues into September and October. With early showers, soil moisture levels may increase, causing new flush development and a significant increase of delicate foliage that is very susceptible to infection. The same circumstance would also cause a lot of root growth, coincide with the accumulation of *P.capsici* propagules in the soil, and create extremely favourable conditions for disease development [20].

6. DISEASE MANAGEMENT

It is necessary to gather plant propagule from a healthy plant and from an uninfected area. Cuttings should be fungicide-treated after being washed to eliminate soil that has adhered to them [21]. Spraying 1% of a Bordeaux mixture during the rainy season was reported to be successful. Either solarization or methyl bromide should be used to sterilise the nursery mixture. Spreading nursery soil, misting it with water, and then covering it with plastic will solarize the soil. Adopting integrated disease management strategies will help to control the

infection [22]. In most of the situations *P.capsici* is typically a soil-borne pathogen and is present in natural ecosystem and it is crucial to repress these pathogens to increase the strength and yield of the vine. In order to increase crop yield and environmental resilience, the most recent genomic developments have quickened breeding and trait development. Biocontrol agents like *Trichoderma harzianum* and *Pseudomonas fluorescens* are used in crops to control the infection [23, 24]. Recently, a farmer from South India, has produced two pepper types that are resistant to quick wilt. The local varieties Uthirankotta and Karimunda served as the female parents for the development of the Ashwati and Suvarna pepper varieties, while Cheruvally served as the male father for both varieties. Both of these two types produce more dry peppers, grow quickly, and are resistant to quick wilt.

7. GENE EDITING IN BLACKPEPPER USING A CRISPR/Cas SYSTEM

Black pepper breeding initiatives should give priority to traits linked to yield stability and sustainability with the current production trends, projected population rise, and environmental concerns. These characteristics include a high rate of fruit set, resilience to biotic and abiotic stress, and stress tolerance. Black pepper gene editing is still in its early phases, with the majority of research concentrating on the identification of important genes regulating numerous beneficial agronomic features. With the help of quick gene-editing technologies, crop types could be improved and stably inherited point changes could be added to the plant genome, leading to non-transgenic plants. This is so that the transgenic region can be easily removed after a gene has been altered. The resistant pathogenic populations contribute to the current limitations in the management of diseases brought on by *P.capsici*. The implementation of an aggressive integrated management strategy may not be enough to control the disease when the climate is conducive to it [25,26]. In modern days cross breeding, transgenic breeding and mutation breeding are the different methods adopted in crop improvement. But it takes a long period to add desirable alleles and to increase genetic variation [27]. Based on current studies on different crops Genome editing emerges a new strategy for crop improvement [28]. Genome editing is a useful technique for controlling *P. capsici* and preventing economic losses from the diseases it causes. In recent times, efficient gene editing technologies have been developed [29]. Sequence specific nucleases such as Mega nucleases, Zinc finger nucleases,

Transcription activator-like effector nucleases and Cas proteins are the different genome editing technologies used. Despite the fact that use of these two technologies made an immense impact on crop improvement but there are certain limitations which paves the way for CRISPR/Cas system. The CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) has emerged in 2013 as a result of studies in rice (*Oryza sativa*), wheat (*Triticum aestivum*), *Nicotiana benthamiana*, and *Arabidopsis thaliana*. It was a great impact for the plant breeders which delivers an immense revolutionary tool for the fast evolution of agricultural crops. These technologies are widely used, affordable, simple-to-use techniques for targeted genetic manipulation that has been used on different crops. Genome editing has altered traits like yield, quality, and tolerance to biotic and abiotic stress [30]. This strategy has also improved hybrid breeding methods, making it easier to modify crop features precisely, even within a single generation, by removing undesirable traits or introducing desired traits to superior types. Thus, CRISPR/Cas has the potential to improve environmental sustainability and worldwide food production [31].

7.1. CRISPR/Cas SYSTEM

A recently developed technique CRISPR/Cas system is evolved from the bacterial and archae that protects from phages and also it cleaves harmful invader's nucleic acid genome [32]. It is a defense mechanism that allows for precise genome editing. CRISPR/Cas consists of short repeating spacer arrays which is transcribed into CRISPR RNAs (crRNA) and tracker RNAs (tracRNA) and also some Cas genes with endonuclease activity [33]. When foreign genetic elements infect prokaryotes, Cas proteins can cleave the invaders' DNA into small fragments, which are subsequently incorporated into the CRISPR array as new spacers. Repeated invasions of the same invader are rapidly identified by crRNA, which pairs with the foreign DNA to induce Cas protein to break target foreign DNA sequences thus safeguarding the host [34]. CRISPR/CAS Systems have been divided into two classes. Based on Cas genes these two classes subdivided into six types. This division is based on effector cas proteins which imparts immunization by cleaving alien nucleic acid [35]. Types I, III, and IV are the class I systems CRISPR/Cas which use multi-Cas protein complexes for interference. While class 2 systems (types II, V, and VI) use a single effector protein for interference in conjunction with CRISPR RNAs [36]. The most common method is type II

CRISPR/Cas which is isolated from *Streptococcus pyogenes* (SpCas9) [37]. It consists of Cas9 nuclease and guide RNA (gRNA) [38]. A double-strand break is produced when gRNA selectively binds to the target sequence found in genomic DNA and leads Cas9 to a target site for cleavage [39]. Cas 9 consists of HNH and RuvC like domain and each of them cleaves the double stranded DNA. A single guide RNA is a fusion of CRISPR RNA and tracker RNA. The Cas9 protein must bind to the target DNA via a protospacer adjacent motif (PAM) sequence [40].

7.2. Genome editing via CRISPR-induced DNA double-strand breaks

The generation of DNA double-strand breaks (DSBs) at target loci, which can be utilized to introduce a variety of genomic alterations via one of the two main DNA repair pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR) is a crucial feature of the CRISPR/Cas gene editing technique [41].

7.3. Genome editing by non-homologous end joining

NHEJ is an error prone mechanism in which a homologous template is need not necessary. It is the most common method which creates small deletions or insertions that disrupts specific points in target genes [42]. When compared with other nucleases such as TALENs or zinc finger nucleases, CRISPR systems is better in which it can target multiple sites together with multiple sgRNAs with an expression of single Cas9 protein [43]. Different Gene knockout studies are done using this method.

7.4. Genome editing via the homology-directed repair pathway

NHEJ is extremely effective and ideal for large-scale knockout research, but it lacks the accuracy needed for more complex genome editing. It is possible to accurately introduce desired sequences into the target DNA and insert or replace specific point mutations using HDR-mediated genome editing [44]. The cell cycle's S- and G2-phases are where HDR starts. A template with homology to the break site is needed for DSB repair [45]. The sister chromatid or an exogenous template, such as exogenous DNA or single-strand DNA, that has the desired sequence alteration to be inserted into the break site, can serve as the repair template. Numerous organisms have exploited precise HDR-mediated

genomic editing extensively [46]. Due to HDR's low effectiveness and the constraints of donor template distribution in plant cells, it is still quite difficult to execute HDR-mediated gene targeting in plants. There have been numerous methods employed to enhance HDR-mediated gene targeting in plants.

8. STEPS INVOLVED IN CRISPR/Cas GENE EDITING

8.1. Gene targeting and single guide RNA designing

Eukaryotic translation initiation factors (eIFs), such as eIF4E, eIF4G, and similar proteins, are encoded by a large number of the genes in plants that are recessive to pathogens. Importantly, eIF4E and its isoform, eIF(iso)4E, play critical roles in viral and fungal infection and function as recessive resistance genes against a variety of pathogens in a variety of plants [47]. Hence, the initial step is to find and target these recessive resistant genes in blackpepper. After gene identification gRNAs are designed.

8.2. Single guide RNA synthesis and cloning

Guide RNAs are created particularly to point Cas9 at the desired target gene for editing. The gRNAs are then constructed using a variety of software programmes, like Benchling, CRISPR-P, CRISPR-PLANT [48], CRISPR direct [49], Chop-Chop, and CRISPRdirect [50]. These gRNAs were cloned in a binary vector to make colonies. So a CRISPR vector search in plasmid libraries like Addgene should be finished in order to put the gRNAs and CRISPR/Cas9 cassette together. Software like Benchling and Snapgene can be used to simulate the vector construction process.

8.3. Multiplex gene target

The multiplexing ability of CRISPR-Cas9 is a significant extension. There are currently no credible methods to precisely predict the effectiveness of a single gRNA in vivo. Hence, numerous gRNAs can be employed to simultaneously target various loci of a single gene in order to ensure successful gene editing. Assembling multiple gRNA transcription units head to tail in a binary vector that also contains a Cas9 gene expression cassette is the standard method for CRISPR/Cas9 multiplexing. Each gRNA transcription unit consists of a gRNA, a scaffold sequence for the gRNA, an RNA polymerase (Pol) III promoter, such as the rice U3 or Arabidopsis U6 small nuclear RNA (snRNA) promoter, and a U3 or U6

terminator sequence. A group of genes called snRNAs have a role in pre-mRNA splicing in plants. These U3 or U6 snRNA promoters can produce relatively large quantities of an RNA transcript because they are constitutively expressed. Based on this method, several efficient cloning vectors were created that simply need the gRNA sequence(s) to be inserted into the cassette [51].

8.4. Delivery method for host system

Key steps in genome editing include the introduction of editing agents to plant cells and the generation of editing events. *Agrobacterium*-mediated transfer DNA (T-DNA) transformation, protoplast transfection, and particle bombardment are three methods for introducing CRISPR-mediated editing reagents, such as DNA, RNA, and ribonucleoproteins (RNPs), which is incorporated into plant cells [52]. The two main delivery strategies for the creation of altered plants are *Agrobacterium*-mediated transformation and particle bombardment [53]. For the transformation of watermelons, pHSN401, pHSN501, and pHSE401 are utilised [54]; for the transformation of tomatoes, pTC217 is employed [55]. Both ligation-dependent [56, 57] and ligation-independent [58] procedures can be used to create the CRISPR constructs, which are then sequenced to ensure appropriate alignment.

8.5. Screening and conformation of transgenics

Among all the molecular techniques used to confirm the transgene, the polymerase chain reaction (PCR) approach is one of the most accurate and straightforward. Primers are typically employed in PCR that are specific to the gene of interest and the site of plasmid constructs used to create transgenic plants. Successful amplification of the DNA fragment with the anticipated band suggests the potential presence of a transgene, and DNA sequencing is used to confirm this DNA fragment. A real-time PCR delivers quick, sensitive, and high-throughput molecular PCR-based analysis compared to the classical Southern blot analysis especially in the area of transgene copy number and zygosity detection in transgenic plants [59].

8.6. Evaluation for biotic stress tolerance

It is the phenotypic evaluation of CRISPR/Cas gene edited black pepper. In order to confirm whether the black pepper shows tolerance to biotic stress.

9. CRISPR/Cas STUDIES ON DIFFERENT TYPES OF CROPS

Recent developments in CRISPR technology have made it possible for researchers to create a wide variety of CRISPR variants with various uses. CRISPR/Cas9 is one of the most widely used genome editing techniques in the plant world. Despite the fast advancement of gene editing, CRISPR/Cas9 remains a reliable, accurate, and frequently employed tool [60]. CRISPR/Cas9-edited crops have demonstrated significant efficacy [61]. Numerous genome efficiencies are among them, with some reaching as high as 91.6% in rice [62] and as low as 79% in maize [63]. CRISPR/Cas9 technology has been used to modify a number of horticulture crops in order to achieve a variety of research goals, such as understanding gene function and a number of applied breeding objectives [64]. The major application of CRISPR/Cas is the gene disruption by deletions in coding sequences which successfully created resistance in *Arabidopsis* and cucumber against a number of RNA viruses [65]. A similar method is gene disruption by deletions in promoter region which created a blight resistance against bacterial blight pathogen [66]. Gene disruption by deleting sgRNAs create large chromosomal deletions which develop lasting resistance against target pathogen [67]. The use of the *OsSWEET* gene to elicit immunity against bacterial blight brought on by *Xanthomonas oryzae* pv. *oryzae* is the most successful example of CRISPR-mediated induction of bacterial resistance in crops [68]. Around 30% of newly emergent plant diseases are caused by fungi, which also affect many commercially significant food crops [69]. Plant S genes have been targeted and disrupted using CRISPR technologies to increase resistance to fungi. Multiple studies have shown that the mutation of Barley Mildew Resistance Locus O (Mlo), which encodes a membrane-associated protein necessary for the fungal pathogen to penetrate the host epidermal cells, results in plant immunity to powdery mildew [70,71]. CRISPR has also been utilized for treating oomycete infection [72]. The papaya plant mutant for a functional cysteine protease inhibitor (PpalEPIC8) was developed using the CRISPR-Cas9 method, increasing the plant's resistance to the destructive oomycete disease against *Phytophthora palmivora* [73].

10. CONCLUSION

In many parts of the Wayanad and Idukki districts of South India, foot rot of black pepper was reported to be a

particularly destructive disease. Currently there is no known effective method for protecting the pepper plants from Quick wilt. Further research is needed to study the different bio-physiological interactions between the pathogen and pepper plant. A recent advance in CRISPR/Cas has become the most crucial tool for molecular biology over the past two years. Therefore, implementing this technique will result in a more thorough comprehension of gene function in plants. Despite the crop's high yielding variety, the traditional breeding process for black pepper takes more time. The breeding season is between ten and twenty years. Regardless of the challenges posed by various diseases and the possible harmful effects of climate change, the demand for black pepper is gradually rising. Enhancing crop characteristics while ensuring crop output stability and sustainability are the main objectives of black pepper cultivation.



Fig. 1: Unripe fruit of blackpepper plant (*Piper nigrum*)



Fig. 2: *Phytophthora capsici*, a destructive pathogen in pepper crops

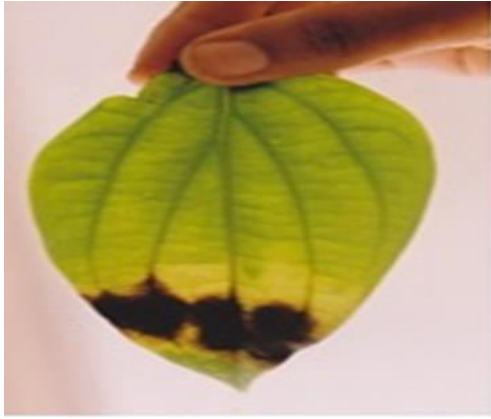


Fig. 3: Quick wilt disease on blackpepper affected by *Phytophthora capsici*

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