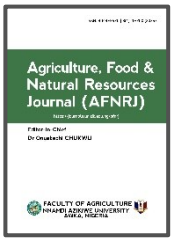
Agr. Foo. Nat. Res. J. Volume 5, Issue 1, Page: 21-35

e-ISSN: 1597-7153 | p-ISSN: 3043-5420

Agriculture, Food and Natural Resources Journal

The Official Journal of the Faculty of Agriculture, Nnamdi Azikiwe University, Awka, Nigeria

Journal homepage: https://journals.unizik.edu.ng/afnrj



Review Article

A relook on the resistance of plant pathogenic fungi to the fungicide Benzimidazoles: What is there to learn?

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DOI: https://www.doi.org/10.5281/zenodo.20129236

Editor: *Dr Onyekachi Chukwu, Nnamdi Azikiwe University, NIGERIA*

Received: *November 22, 2025*
Accepted: *February 7, 2026*
Available online: *March 31, 2026*

Peer-review: *Externally peer-reviewed*

Conflict of Interest: *The authors have no conflicts of interest to declare*

Financial Disclosure: *The authors declared that this study has received no financial support.*

ABSTRACT

Benzimidazoles are systemic fungicides that disrupt fungal energy metabolism by binding with high affinity to β-tubulin, thereby interfering with microtubule assembly. Resistance to this group of fungicides is primarily associated with alterations in the β-tubulin gene that reduce fungicide binding. Single-point mutations, especially at codon 198 (GAG→GCG, resulting in the E198A substitution), are among the most frequently reported mechanisms. Similar substitutions at other amino acid positions in β-tubulin have also been linked to resistance in several plant pathogenic fungi, including Botrytis cinerea, Helminthosporium solani, and Tapesia acuformis, as well as in various field-resistant populations. In Fusarium fujikuroi, resistance has been associated specifically with mutations in the β2-tubulin (β2tub) gene rather than β1-tubulin (β1tub). A Tyr50Asp substitution in β1-tubulin has been shown to confer resistance in UV-induced mutants of Fusarium moniliforme. Additional mutations such as Phe167Tyr, Glu198Ala, Glu198Val, Glu198Gly, and Phe200Tyr have been reported in F. graminearum, Helminthosporium solani, and other phytopathogenic fungi, further demonstrating the diversity of resistance-associated substitutions within the β-tubulin gene. In F. asiaticum, a point mutation at codon 198 in the β2-tubulin gene has been strongly correlated with high levels of resistance to carbendazim. Removal of the fourth intron in β2tub increases sensitivity to carbendazim in F. graminearum, whereas deletion of the first and second introns enhances β2-tubulin protein expression and consequently reduces sensitivity. This review therefore examines the molecular basis and diversity of resistance mechanisms to benzimidazoles among economically important plant pathogenic fungi.

KEYWORDS: *Benzimidazole Fungicides resistance, β-tubulin, Inhibitors, Mutation, Plant pathogenic fungi*

INTRODUCTION

Benzimidazoles are an important class of systemic fungicides marketed under active ingredients such as benomyl, carbendazim (methyl benzimidazol-2-yl carbamate, MBC), thiophanate-methyl, thiabendazole, and fuberidazole (Leadbeater, 2014). They exhibit broad-spectrum activity against numerous fungal pathogens, particularly ascomycetes, certain basidiomycetes, and deuteromycetes (Leadbeater, 2014). In agricultural practice, these fungicides are widely applied to cereals, fruits, vegetables, and grapevines and they are also used in postharvest disease management (Duan *et al.* 2019; Leadbeater 2014; Oliver and Hewitt 2014). They are effective against pathogens including species of *Cercospora* and *Fusarium*, as well as *Botrytis cinerea*, *Colletotrichum* species, and powdery mildew fungi such as *Erysiphe* and *Oidium* species (Duan *et al.,* 2019; Leadbeater 2014). The extensive use of benzimidazoles is largely due to their strong inhibitory activity, selective mode of action, and the pharmacological versatility of the benzimidazole heterocyclic

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nucleus (El-masry *et al.,* 2000; Hosamani and Shingalapur 2011; Navarrete-Vázquez *et al.* 2006; Zhou *et al.* 2016a). Beyond agriculture, benzimidazole derivatives are known for diverse biological activities, including anticancer, antiviral, antibacterial, antifungal, anthelmintic, anti-inflammatory, antihistaminic, antioxidant, antihypertensive, anticoagulant, and proton pump inhibitory effects (Tuncbilek *et al.* 2009; Zhou *et al.* 2016a). This broad bioactivity profile highlights the structural and functional importance of the benzimidazole scaffold. Physicochemical, benzimidazole fungicides have limited solubility at neutral pH but become more soluble under acidic conditions. Among them, carbendazim, thiabendazole, and fuberidazole are systemic, enabling translocation within plant tissues (Oliver & Hewitt 2014; Roberts *et al.* 2007). In contrast, benomyl and thiophanate-methyl primarily act as contact fungicides; however, both can be metabolically converted to carbendazim in soil environments and are readily degraded by soil microorganisms. Additionally, these compounds may undergo hydrolytic and photolytic degradation within plant tissues. Toxicological profiles differ slightly among members of this group. Benomyl and carbendazim are generally considered to have relatively low toxicity, whereas fuberidazole exhibits moderate toxicity. Carbendazim remains the most extensively used benzimidazole fungicide worldwide, particularly in the management of diseases affecting cereals and fruit crops.

The mode of action of benzimidazole fungicides including; carbendazim, benomyl, and thiabendazole centers on their ability to disrupt fungal microtubule formation by inhibiting tubulin polymerization (Zhou *et al.,* 2016a). Experimental approaches such as turbidity assays and homology modeling have been employed to elucidate their interactions with fungal tubulin and to Benzimidazole fungicides have played a major role in the management of numerous plant pathogenic fungi and have been widely used since their introduction, despite the emergence of resistance problems beginning in the early 1970s (Leadbeater 2014; Zhou & Jia 2015). Many fungal pathogens can rapidly develop resistance to this group of fungicides, sometimes within two to four growing seasons (Brent & Hollomon 2000; List 2017; Oliver & Hewitt 2014). However, their effectiveness can be prolonged when they are applied in mixtures or rotation programs with fungicides that have different modes of action. Resistance development varies among pathogens. Species of *Botrytis* readily produce resistant populations to benzimidazoles, whereas resistance in *Oculimacula* species was reported only after approximately a decade of continuous use. In France, between 1997 and 2003, numerous benzimidazole-resistant isolates of *Mycosphaerella graminicola* and *Oculimacula spp*. were recovered from wheat fields (Zhou & Jia 2015). Similarly, resistance in *Corynespora cassiicola* was documented in Japan (Date *et al.* 2004; Ishii *et al.* 2007).

To mitigate resistance, the use of alternative fungicides or mixtures with different modes of action has been strongly recommended (List, 2017). Diethofencarb, for example, exhibits negative cross-resistance with benzimidazoles, meaning that strains resistant to benzimidazoles may remain sensitive to diethofencarb (Zhou & Jia 2015). Consequently, mixtures such as carbendazim combined with diethofencarb were considered effective against *Botrytis cinerea*, although the widespread use of both fungicides eventually led to additional resistance challenges (Leroux & Fritz 1984). Benzimidazoles exert their antifungal activity by disrupting cellular division. They bind selectively and with high affinity to β-tubulin in fungal cells, inhibiting microtubule assembly. This interference prevents proper chromosome segregation during mitosis, ultimately leading to cellular dysfunction and death. Because of this mechanism, benzimidazoles are particularly effective against a broad range of plant pathogenic fungi, including *Botrytis cinerea, Cercospora spp*., *Colletotrichum spp., Fusarium spp., Erysiphe spp.,* and *Oidium spp* (Davidse 1995; Davidse 1986; Duan *et al.* 2019; Leadbeater 2014; Oliver & Hewitt 2014). In contrast, oomycetes and plants are inherently insensitive to benzimidazoles (Davidse 1986). Resistance to benzimidazole fungicides has profound effects on key physiological processes in fungi, including spore germination, vegetative growth, cell multiplication, and mitotic division (Committee 2016; List 2018). The primary mechanism underlying this resistance involves alterations in the β-tubulin protein, which reduce the fungicide's ability to bind effectively to its target site (Davidse and Flach 1978; Duan *et al.* 2019; Koenraadt and Jones 1993; Zhou *et al.* 2016a; Zhou *et al.* 2016b). Benzimidazoles normally interact with β-tubulin with high affinity, disrupting microtubule assembly and thereby inhibiting cell division. However, mutations in the β-tubulin gene modify the protein structure and prevent efficient fungicide binding. Numerous mutations have been identified at specific codons of the β-tubulin gene, including positions 6, 50, 167, 198, 200, and 240 (Duan *et al.* 2019; Hawkins and Fraaije 2016). Among these, substitutions at codons 198 and 200 are the most frequently reported and are strongly associated with resistance in many plant pathogenic fungi. For example, the substitution at codon 198 (GAG→GCG, resulting in Glu198Ala or E198A) has been widely documented in resistant populations of *Botrytis cinerea*, *Helminthosporium solani*, and *Tapesia acuformis*, as well as in other field isolates (Albertini *et al.* 1999; Chen *et al.* 2014; Duan *et al.* 2015; Luck & Gillings 1995; MKay & Cooke, 1997).

In *Fusarium fujikuroi*, resistance has been linked specifically to mutations in the β2-tubulin gene rather than β1-tubulin, including Glu198Val, Phe200Tyr, and Gly235 alterations (Chen *et al.* 2014). Similarly, a Try50Asp substitution in β1-tubulin has been associated with resistance in UV-induced mutants of *F. moniliforme* (Yan & Dickman 1996). Additional amino acid changes such as Phe167Tyr, Glu198Ala/Val/Gly, and other substitutions within the β-tubulin coding region have been identified in resistant strains of *F. graminearum*, *Helminthosporium solani*, and several other phytopathogenic fungi (Albertini *et al.* 1999; Chen *et al.* 2014; Koenraadt *et al.* 1992; Leroux *et al.* 2002; Ma *et al.* 2005). In *F. asiaticum*, the E198 mutation in the β2-tubulin gene confers a high level of

resistance to carbendazim without imposing a detectable fitness penalty (Yang *et al.* 2018). Structural variations in the β2-tubulin gene can also influence sensitivity. For instance, deletion of the fourth intron increases sensitivity to carbendazim in *F. graminearum* (Li *et al.* 2017), whereas removal of the first and second introns enhances β2-tubulin protein production and reduces fungicide sensitivity. Furthermore, phenylalanine at position 240 (F240) of β2-tubulin in *F. graminearum* contributes to reduced binding affinity between carbendazim and the target protein when compared with other fungi such as *B. cinerea*, *Colletotrichum gloeosporioides*, and *Sclerotinia sclerotiorum* (Zhu *et al.* 2018). Beyond target-site mutations, alternative mechanisms may also contribute to resistance (Yang *et al.* 2019). In *Isaria fumosorosea*, elevated resistance to carbendazim has been attributed to the overexpression of ATP-binding cassette (ABC) transporters rather than changes in the β-tubulin gene. These transporters likely enhance fungicide efflux, thereby decreasing intracellular fungicide concentration (Song *et al.* 2012). Resistance can also have broader biological consequences. Studies investigating pleiotropic effects have shown that benzimidazole-resistant strains may produce higher levels of certain mycotoxins, such as patulin and citrinin, compared with sensitive strains in apple fruit (Zhang *et al.* 2009; Malandrakis *et al.* 2013).

Additionally, benzimidazole resistance has been associated with increased trichothecene production in *F. graminearum* (Yang *et al.* 2019; Zhu *et al.* 2018). At the molecular level, these resistance phenomena are closely linked to point mutations that alter amino acids within or near the benzimidazole binding site of β-tubulin, thereby reducing fungicide affinity (Song *et al.* 2012). Due to the rapid selection of resistant populations and the widespread occurrence of target-site mutations, the Fungicide Resistance Action Committee (FRAC) classifies benzimidazoles as high risk for resistance development (FRAC 2017). A comprehensive understanding of their mode of action, resistance mechanisms, and field applications is therefore essential. Such knowledge supports the development of improved resistance management strategies, including early detection and monitoring programs, the design of novel compounds, and integrated approaches for sustainable plant disease control.

Briefly, this review covers:

- Mode of actions
- Benzimidazole resistance
- Mechanism of resistance
- Method of detection

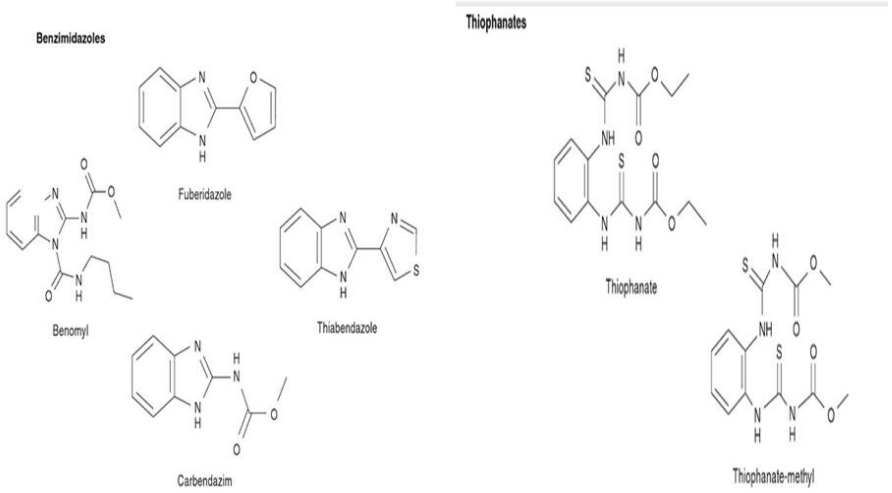

Figure 1: Chemical structures of Benzimidazole (Oliver & Hewitt 2014)

MECHANISM OF ACTION

Inhibition of β-tubulin polymerization

Benzimidazole fungicides are highly effective inhibitors of β-tubulin polymerization in fungal cells. By binding to β-tubulin and disrupting microtubule assembly, they interfere with mitosis and prevent normal cell division. This targeted mode of action makes them particularly valuable for controlling a wide range of plant-pathogenic fungi. As a result, benzimidazoles have played an important role in plant disease prevention and management programs, especially when incorporated into integrated disease control strategies (Committee, 2013; Zhou *et al.* 2020).

Direct interactions of inhibitors with β-tubulin and the interactions with other forms of tubulins were responsible for the polymerization (Zhou *et al.* 2020). The β_2-tubulin of *F. graminearum* was the target of benzimidazole products obtained through purification of the recombinant β2-tubulins (Zhou *et al.* 2016a). B2-tubulin gene binds to α_2-tubulin while β_1-tubulin binds to α_1-tubulin gene. Little changes were seen in A_{350} polymerization mixtures of α_1-/ β2-tubulin and α_2-/ β2

when carbendazim was added to the mixture before polymerization showing that carbendazim significantly inhibited the polymerization of tubulins (Zhou *et al.* 2016a). Therefore, benzimidazole compounds (Table 1) interfere with the polymerization of monomeric tubulins and not polymerized microtubules thus disturbing the microtubule dynamic (Zhou *et al.* 2016a). Increase inhibition rate of the three fungicides; carbendazim, benomyl and thiabendazole on *F. graminearum* decrease the EC_{50}. They interact with β-tubulin and stopped mycelial growth in fusarium head blight (Hollomon *et al.* 1998).

Table1. Effects of benzimidazole compounds on *Fusarium graminearum* and tubulin polymerization *in vitro*,

Benzimidazole compounds	Inhibition ratio of α_1 and β_2-tubulin polymerization (%)	Inhibition ratio of α_2 and β_2-tubulin polymerization (%)	EC_{50} (µM)
Carbendazim	90±0.4	93.5±0.05	2.46
Benomyl	89.9±0.1	92.6±1.2	2.1
Thiabendazole	81.6±1.0	20.1±1.9	5.61

Source (Zhou *et al.* 2016a).

Benzimidazole disrupts mitosis

Benzimidazoles interfere with both mitotic and meiotic cell division in plant and animal cells. They act by binding to β-tubulin, thereby preventing proper microtubule formation. This disruption impairs the assembly and function of the spindle apparatus, particularly at metaphase, leading to the breakdown of the bipolar microtubule structure required for accurate chromosome segregation. As a result, normal cell division is inhibited (Zhou *et al.* 2016a). This leads to the failure of segregation by the daughter cells and consequently resulting to death of the cell (Davidse 1986). Carbendazim exerts its antifungal activity by interfering with mitosis. It binds specifically to β-tubulin, disrupting microtubule formation and impairing normal spindle development during cell division. By inhibiting proper tubulin assembly and function, it effectively blocks mitotic progression. Owing to this well-defined mechanism of action, carbendazim has also been widely used as a reference compound in the screening and evaluation of potential antimitotic agents (Davidse 1986). Microtubules are polymers of tubulins that form part of cytoskeleton which provide structure and shape to eukaryotic cells and are active in spindle formation and the separation of chromosomes in cell division(Zhou *et al.* 2016a).

Selectivity

Although β-tubulin is highly conserved among eukaryotic organisms, benzimidazoles exhibit a remarkable degree of selectivity in their biological activity. This selectivity arises from subtle structural differences in the β-tubulin binding sites that influence the affinity of these fungicides for their target. As a result, benzimidazoles preferentially affect certain fungal groups while having limited or no impact on others. Microtubules are present in all eukaryotic fungi; however, the sensitivity to benzimidazoles varies considerably among taxa. Carbendazim, for example, shows strong activity against many ascomycete fungi, reduced effectiveness against basidiomycetes, and little to no activity against oomycetes, zygomycetes, higher plants, or animals. This differential response reflects variations in β-tubulin structure that determine fungicide binding efficiency and, consequently, biological activity (Davidse 1986; Zhou *et al.* 2020). Benzimidazoles also demonstrate selective therapeutic activity in animals, particularly in the treatment of parasitic worm infections such as tapeworms. Their selectivity within eukaryotic organisms is largely determined by differences in the binding affinity of individual benzimidazole compounds to tubulin in various species, as well as variations in metabolic processing. In host–parasite systems, this differential affinity plays a crucial role. Anthelmintic benzimidazoles, for example, bind more strongly to the tubulin of parasitic worms than to that of the host animal. This preferential binding disrupts microtubule formation in the parasite while causing minimal effects on host tissues. Consequently, variations in tubulin structure and metabolic pathways among organisms underpin the selective toxicity and therapeutic usefulness of benzimidazoles (Friedman and Platzer 1980; Morejohn and Fosket 1984).

Binding affinity and in vitro inhibition of microtubule assembly

Binding studies (Davidse 1986; Zhou *et al.* 2016a) involving carbendazim and crude extracts of *Aspergillus nidulans* demonstrated that the biological activity of benzimidazoles depends largely on their affinity for specific tubulin proteins. In these experiments, carbendazim showed selective interaction with fungal tubulin, supporting the conclusion that target-site binding is central to the mode of action of benzimidazole compounds. These findings highlight that the strength and specificity of tubulin binding largely determine the antifungal effectiveness of this class of fungicides. The degree of carbendazim binding to tubulin correlates closely with the sensitivity of a given fungal strain (Table 2). Studies have shown that strains exhibiting stronger binding affinity tend to be more susceptible, whereas reduced binding is associated with resistance (Davidse 1986; Friedman and Platzer 1978; Hoebeke *et al.* 1976). Competitive binding experiments using radiolabeled (14 ^{0}C) carbendazim further demonstrated that colchicine and nocodazole can inhibit carbendazim attachment to fungal tubulin. This competition confirmed that tubulin functions as the primary carbendazim-binding protein in fungi (Howard 1977; Howard & Aist 1980). Differences in tubulin sensitivity among organisms also help explain the selectivity of benzimidazoles. In mammalian systems, microtubule assembly is strongly inhibited by colchicine and nocodazole, while carbendazim exerts only minor effects. In contrast, yeast tubulin assembly is inhibited by both nocodazole and carbendazim, and sensitivity increases when the two compounds are combined. These in vitro findings support the conclusion that the

cytological effects of benzimidazoles in fungi result from disruption of normal microtubule assembly *in vivo*. Microscopic studies on hyphal cells of *F. acuminatum* have confirmed that carbendazim alters spindle formation and microtubule organization, leading to impaired cell division (Zhou *et al.* 2016a). Mechanistically, benzimidazoles inhibit microtubule polymerization by interfering with the addition of tubulin subunits to the growing ends of microtubules. When tubulin associated with colchicine is incorporated into a microtubule, it reduces the ability of subsequent tubulin molecules to attach, thereby halting further elongation. In *F. acuminatum*, β1-tubulin has been identified as the primary binding target of carbendazim, whereas β2-tubulin does not appear to play a major role in this interaction. Together, these findings emphasize that selective binding to specific tubulin isoforms underlies both the antifungal activity and species-specific effects of benzimidazole fungicides (Zhou *et al.* 2016b).

Table 2: the sensitivity to carbendazim and the dissociation constant of the carbazim-tubublin complex in strain of *Aspergillus nidulans*

Strain	Mutation	EC_{50} value against growth (µM)		Dissociation constant of the carbendazim-tubulin complex (µM)
		Carbedazim	nocodazole	
3	_____	4.5	0.5	2.2
186	*benA16*	1.5	0.23	0.6
R	*benA15*	95	20	27

Source: (Davidse 1986).

Introns mediated regulation of β-tubulins genes in Benzimidazoles sensitivity

The introns of β-tubulins genes can regulate benzimidazole fungicide sensitivity by influencing the expression of β-tubulins genes(Li *et al.* 2017). The target genes of benzimidazole fungicides in human and fungi are the β-tubulins. β_1 tub and β_2 tub gene are remarkably observed in *F. graminearum* (Zhao *et al.* 2014). Modification of intron regions within the β2-tubulin gene has been shown to influence carbendazim sensitivity in *F. graminearum*. Removal of the fourth intron increases the fungus's susceptibility to carbendazim. In contrast, deletion of the first and second introns enhances the expression of β2-tubulin, resulting in greater protein production and a corresponding reduction in sensitivity to the fungicide. These findings indicate that intron structure can regulate β2-tubulin expression levels and thereby affect the response of the fungus to carbendazim (Li *et al.* 2017). B2-tubulin plays a crucial role in the vegetative growth of *F. graminearum*. Deletion of the β2-tubulin gene leads to significant reductions in growth rate, sporulation, and virulence. Consequently, β2-tubulin mutants also display altered sensitivity to carbendazim, highlighting the importance of this protein in both fungal development and fungicide response (Qiu *et al.* 2011). Further studies have shown that β1-tubulin plays a positive role in regulating carbendazim sensitivity in *F. graminearum*. Analyses using quantitative real-time PCR (qRT-PCR) and Western blotting revealed that the expression levels of the β1-tubulin gene were significantly upregulated, indicating that higher β1-tubulin levels enhance the fungus's susceptibility to carbendazim (Li *et al.* 2017). Furthermore, deletion of β_1-tub in *Gibberella zeae* inhibits the mycelial growth and virulence but accelerates vegetative reproduction (Qiu *et al.* 2011).

MECHANISMS PATHOGENIC FUNGI RESISTANCE TO BENZIMIDAZOLE

Structural alterations in the target site

Structural alteration in the α-tubulin gene decreases sensitivity of pathogens to benzimidazoles thus reduce the fungicide affinity (Ma and Michailides, 2005). Point mutations in β-tubulin genes (Goldman *et al.* 1993) cause carbendazim resistance by changing the amino acids in the carbendazim binding site. Fungicidal action and resistance depend on the compatibility of specified inhibitors for chosen area on the β-tubulin protein (Zhou *et al.* 2016a), Resistance to benzimidazole fungicides arises primarily from specific alterations within the β-tubulin binding site. In particular, carbendazim resistance linked to β2-tubulin substitutions reduces the binding interaction between β2-tubulin and IDH3 (isocitrate dehydrogenase subunit 3). Molecular studies have further confirmed that point mutations in the β2-tubulin gene (FGSG_06611.3) are responsible for conferring benzimidazole resistance in *F. asiaticum*, highlighting the critical role of these genetic changes in determining fungicide sensitivity (Chen *et al.* 2009; Duan *et al.* 2016a; Li *et al.* 2003; Qiu *et al.* 2011; Yuan & Zhou 2005). Mutations conferring benzimidazole resistance have been identified at specific sites within the β-tubulin gene, notably at codons 167, 198, and 200. Alterations at these positions change the amino acid sequence of β-tubulin, disrupting fungicide binding and thereby reducing the sensitivity of the fungus to benzimidazole compounds (Duan *et al.* 2016a). Specific amino acid substitutions in β-tubulin are key determinants of benzimidazole resistance. At codon 198, the replacement of glutamic acid (Glu) with alanine (Ala), glycine (Gly), or valine (Val) results in a high level of resistance to benzimidazole fungicides. Similarly, at codon 200,

substitution of phenylalanine (Phe) with tyrosine (Tyr) confers an intermediate level of resistance. These targeted changes alter the fungicide binding site, reducing its effectiveness (Albertini *et al.* 1999; Fujimura *et al.* 1992; Yarden and Katan 1993). In *A. nidulans*, benA mutant's exhibit altered binding affinity of tubulin for benzimidazoles. These changes in fungicide sensitivity are associated with modifications in the primary isoforms of β-tubulin, indicating that structural variation in β-tubulin directly affects the interaction with benzimidazole compounds (Sheir-Neiss *et al.* 1978). Two-dimensional gel electrophoresis analyses of β-tubulin from 26 *ben A* mutants, including benA15 and benA16, revealed that 18 of these mutants exhibited alterations in β-tubulin. These changes were evident in shifts in isoelectric point, variations in electrophoretic mobility on SDS-PAGE, or differences in the relative abundance of β-tubulin isoforms. This demonstrates that mutations in *benA* can substantially modify the structural and biochemical properties of β-tubulin (Davidse 1986). In *Trichoderma* spp. and *Colletotrichum* spp., two distinct β-tubulin genes, tub1 and tub2, have been identified. Reverse genetics studies have demonstrated that mutations in the tub2 gene confer resistance to carbendazim, indicating that tub2 serves as the primary target of this fungicide(Goldman *et al.* 1993). Specifically, a substitution at codon 198 of the tub2 gene has been linked to carbendazim resistance in *C. gloeosporioides* (Kongtragoul *et al.* 2011).

Pleiotropic effects of mutations to resistance

Resistance mutations have harmful effects on the phenotypical characteristics of benzimidazole resistant isolates including sensitivity to temperature, fitness, competiveness, virulence, survival, reproductive and toxins production (Ishii and Holloman, 2015; Malandrakis *et al.,* 2013; Yang *et al.,* 2019). Temperature sensitivity is a notable phenotypic characteristic of benzimidazole-resistant fungal isolates. Mutations in the β-tubulin gene can produce pleiotropic effects, altering fungal growth and development under both high and low temperature conditions. This indicates that changes conferring fungicide resistance may also impact the organism's ability to adapt to environmental stress (Davidse 1986; Yang *et al.* 2019). Benzimidazole-resistant isolates can lose their resistance when exposed to extreme temperatures. Both low and high temperature conditions have been shown to reduce the effectiveness of the resistant strains, indicating that environmental factors can influence the stability and robustness of benzimidazole resistance (Ma *et al.* 2005; Ma *et al.* 2003; Yang *et al.* 2019). In laboratory studies, low-resistant fungal isolates displayed pronounced sensitivity to high temperatures. For example, they were inhibited by 1 μg ml^{-1} of benomyl at 28 °C, whereas they remained resistant at lower temperatures ranging from 8–24 °C. These isolates also exhibited very high resistance to carbendazim but showed low thermotolerance, highlighting the impact of temperature on the expression and stability of benzimidazole resistance (Song *et al.* 2012; Zhang *et al.* 2010; Zou *et al.* 2006). In contrast, *Cercospora beticola* strains with high levels of carbendazim resistance exhibited no noticeable sensitivity to temperature. This suggests that, unlike low-resistant isolates, their resistance remains stable across a range of environmental temperatures (Trkulja *et al.* 2013). Both carbendazim-resistant and -sensitive field strains of *B. theobromae* were found to be sensitive to low temperatures, yet they exhibited tolerance to high temperatures up to 40 °C. This indicates that, while low temperatures can limit their growth, these strains can withstand relatively high thermal conditions without loss of viability (Yang *et al.* 2019). Mutant genes in *A. nidulans* exhibited pleiotropic effects, causing growth inhibition at high temperatures (ts^{-}) and/or low temperatures (cs^{-}). These mutations also increased the fungus's sensitivity to other antimicrotubule agents, demonstrating that alterations in β-tubulin can simultaneously affect temperature tolerance and responsiveness to multiple microtubule-targeting compounds (Davidse 1986). Carbendazim-resistant field isolates of various fungal species often retain sufficient parasitic fitness, allowing them to compete effectively with sensitive strains under natural field conditions. This indicates that resistance does not necessarily compromise the pathogen's ability to infect hosts and reproduce (Ishii and Holloman 2015; Liu *et al.* 2016; Malandrakis *et al.* 2012; Yang *et al.* 2019). Studies investigating the pleiotropic effects of β-tubulin mutations have shown that benzimidazole resistance can influence mycotoxin production. Malandrakis *et al.* (2013) reported that benzimidazole-resistant strains produced significantly higher levels of patulin and citrinin compared with sensitive strains in apple fruit. Similarly, Zhang *et al.* (2009) observed that resistance to benzimidazoles enhances trichothecene production in *Fusarium graminearum*, suggesting that fungicide resistance can have broader metabolic consequences in pathogenic fungi.

Benzimidazoles resistance increases deoxynivalenol biosynthesis (DON)

Resistance to methyl benzimidazole carbamate (MBC) fungicides has been linked to increased production of secondary metabolites, including deoxynivalenol (DON), the most prevalent mycotoxin associated with Fusarium head blight, which poses carcinogenic risks to humans and animals (Audenaert *et al.* 2010; Goswami and Kistler 2004; Tang *et al.* 2018; Van De Walle *et al.* 2010; Zhang *et al.* 2009).. In MBC-resistant mutants of *F. graminearum*, the expression of isocitrate dehydrogenase subunit 3 (IDH3) is significantly reduced due to weakened interactions between β2-tubulin and IDH3. This reduction disrupts normal tricarboxylic acid (TCA) cycle function, leading to the accumulation of acetyl-CoA, a key precursor in DON biosynthesis (Israelsen and Vander Heiden 2015; MacDonald *et al.* 2013; Nitschke and Russell 2013). IDH3 normally acts as a negative regulator of DON production by limiting acetyl-CoA availability, which is predominantly generated in the mitochondrial matrix via the TCA cycle. Research in metabolic engineering has further demonstrated that altering the balance of intracellular acetyl-CoA production and consumption can directly stimulate DON synthesis (Chen *et al.* 2013; Krivoruchko *et al.* 2013; Shiba *et*

al. 2007). In carbendazim-resistant strains, substitutions in β2-tubulin diminish its binding to IDH3, lowering IDH3 levels and causing cytosolic accumulation of acetyl-CoA. This metabolic shift drives enhanced DON biosynthesis, linking fungicide resistance at the molecular level with increased mycotoxin production in *F. graminearum* (Zhou *et al.* 2020).

Mediated regulatory role of introns influence resistance to carbendazim

Introns play a significant role in the evolution and functional divergence of the two β-tubulin genes in *F. graminearum*. By influencing gene structure and expression, introns can regulate the levels of β-tubulin produced, which in turn affects the fungus's sensitivity to carbendazim. Variations in intron composition or splicing may therefore modulate both the functional specialization of β-tubulin isoforms and the degree of fungicide resistance (Li *et al.* 2019; Li *et al.* 2017). Introns regulate fungicide sensitivity in *F. graminearum* by modulating the expression of their corresponding β-tubulin genes (Li *et al.* 2017).. Studies have shown that the loss of introns in β2-tubulin isotypes can alter transcription levels, leading to changes in protein production and, consequently, affecting the fungus's sensitivity to carbendazim (Chen *et al.* 2009). This demonstrates that intron structure plays a key role in controlling β-tubulin expression and fungicide response. Sequence analyses have shown that the *F. graminearum* β1-tubulin gene contains more introns and differs in intron positions compared with β2-tubulin and other β-tubulin genes associated with benzimidazole resistance. These divergent intron patterns between β1- and β2-tubulin are believed to be an evolutionary factor contributing to the functional diversification of β-tubulin genes in *F. graminearum* (Li *et al.* 2019). Additionally, specific amino acid residues classified as type 11 variations have been linked to the functional differences between the two β-tubulins, influencing hyphal growth, asexual development, and sensitivity to carbendazim (Li *et al.* 2019; Zhao *et al.* 2014). Introns play a key role in genome evolution by facilitating exon shuffling and alternative splicing, processes that contribute to the functional diversification of genes (Graveley 2001; Maniatis and Tasic 2002; Rodríguez-Trelles *et al.* 2006). In *F. graminearum*, deletion of the first or second intron from the β2-tubulin gene has been shown to reduce the fungus's sensitivity to carbendazim, highlighting how intron structure can directly influence gene function and fungicide response (Li *et al.* 2017).

Reduced chemical attraction

Research on the methods of pathogenic fungi resistance to benzinidazoles were similar to their mode of action which were made possible by the present of resistant mutants of *A. nidulus* (Ishii 2015; Quaranta *et al.* 2012). Three resistant mutants isolated from *A. nidulus* using standard UV or chemical mutagenesis techniques (Hastie & Georgopoulos, 1971) were used to determine the chemical attraction of carbendazim to β-tubulin (van Tuyl, 1977). Study had shown that the resistance to carbendazim was not caused by the reduction of nutrient uptake or increment of metabolic conversion (Davidse, 1986). Mutation of benA15 lead to resistance to both carbendazim and thiabendazole while mutation of benA16 causes super sensitivity to carbendazim and resistance to thiabendazole (Van Tuyl *et al.* 1974). The chemical attraction of carbendazim to benA15-tubulin decreases where its attraction to benA16-tubulin increased when compared to the parent tubulin. That of thiabendazole to A. nidulans β-tubulin was difficult to estimate using (^{14}C) thiabendazole due to strong specific attraction of chemical to constituent of the unrefined exacts (Davidse and Flach 1978). (^{14}C) carbendazim binding to benA16 tubulin was inhibited by thiabendazole than (^{14}C) carbendazim binding to the wild-type tubulin. This study was used in a chemical attraction analysis using a tubulin from Penicillium expansum parent strains that is resistant to thiabendazole but super sensitive to MBC. The attraction of the resistant tubulin changed to opposite directions (Davidse 1986), in thiabendazole, it was lower than the wild –type but reversed in carbendazim where the binding affinity was higher than wild-type(Davidse 1986). Studies on the chemical attraction of parent strains and benzimidazole resistance isolates including *B. cinerea, F. oxysporum, P. brevicompactum, P. corymbiferum, V. nashicola, Alternaria brassicae* and *Pythium irregulare* to benzimidazoles (Davidse 1986) demonstrated that only exacts of the sensitive type had binding effect. Therefore, the affinity of the target site to benzimidazole is used to detect the efficacy of benzimidazole (Davidse 1986).

Overexpression of benzimidazole fungicides target genes

Overexpression of chemical target gene is also one of the mechanisms of benzimidazoles resistance (Xu *et al.* 2019). This resistance mechanism operates in a dose-dependent manner, where elevated expression of the target gene allows the fungus to maintain functionality even in the presence of benzimidazoles. By producing higher levels of the target protein, the fungus can avoid saturation of binding sites, which can also influence the effectiveness of mixtures with other fungicides (Sanglard *et al.* 2009). Up regulation of the β2-tubulin gene contributes to MBC resistance (Yang *et al.* 2015)in *Paecilomyces lilacinus*, with resistant strains showing a fourfold increase in β2-tubulin expression compared with the parental strain. Similarly, in the *Fusarium* species complex, exposure to carbendazim (Xu *et al.* 2019) either in the field or under laboratory conditions induces upregulation of genes encoding glutathione S-transferase (FVER_00097, FVER_08550, and FVER_09899), particularly FVER_08550, in resistant strains relative to the wild type. Such gene expression changes can facilitate the rapid emergence and establishment of carbendazim-resistant populations in natural environments (Xu *et al.* 2019).

Metabolic decomposition and active efflux by fungi

Fungal resistance to benzimidazole fungicides can also result from decreased intracellular fungicide levels. This occurs through the decomposition of secondary metabolites and active efflux mechanisms that remove the compound from the cell. Overexpression of genes encoding detoxification enzymes and

efflux transporters enhances these processes, leading to lower intracellular fungicide concentrations. Such genetic regulation has been linked to benzimidazole resistance in a variety of fungal species (Andrade *et al.* 2000; Liu *et al.* 2015; Xu *et al.* 2019). Cytochrome P450 monooxygenases (P450s) play a key role in the detoxification of benzimidazole fungicides in the *Fusarium* species complex (Črešnar and Petrič 2011), and P450-mediated metabolism has been implicated in the development of pesticide resistance (Karunker *et al.* 2008; Scott 1999). In addition, fungal efflux systems, particularly ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters, regulate intracellular fungicide levels. These transporters actively export toxins and cellular metabolites, thereby modulating fungicide sensitivity and contributing to resistance (Xu *et al.* 2019).

Sensitivity of resistant strains to N-phenylcarbamates and diphenylamine

A negative cross-resistance relationship exists between benzimidazole-resistant mutants and N-phenylcarbamate fungicides, which disrupt cellular and nuclear division by interfering with microtubule function. For example, studies on *Pseudocercosporella herpotrichoides* and *Venturia nashicola* showed that only highly resistant strains of *V. nashicola* were particularly sensitive to N-phenylcarbamates, whereas intermediate and weakly resistant mutants exhibited little or no sensitivity. This demonstrates that cross-resistance patterns can vary depending on the level of benzimidazole resistance (Cavelier and Leroux 1983; Davidse 1986; Leroux and Fritz 1984). In addition, methyl N-(3,5-dichlorophenyl) carbamate suppressed the myecial growth of benzimidazole resistant mutants of B. cinerea, Cerospora beticola, F. nivale and Mycospharella melonis on mycelial growth inhibition assay.

DETECTION OF BENZIMIDAZOLE FUNGICIDES RESISTANCE

Early detection and monitory of resistance problems over large regions are essential in order to control disease and predict fungicide resistance effectively and proffer solution immediately to avoid subsequent damage. Fungicide resistance was widely detected (Table 3) initially through mycelial inhibition analysis using minimum inhibition concentration (MIC), the method is tedious, required substantial time and low efficiency of resistance detection (Duan *et al.* 2014; Jianxin *et al.* 2002; Zhang *et al.* 2009). Recently, polymerase chain reaction (PCR) techniques have been developed to detect benzimidazole resistance in plant-pathogenic fungi based on their underlying molecular mechanisms. These methods allow for rapid identification of resistant strains carrying the E198A mutation in the β-tubulin gene, enabling timely monitoring and management of fungicide resistance in the field (Chen *et al.* 2009; Duan *et al.* 2018b; Hou *et al.* 2011; Luo *et al.* 2009; Zhang *et al.* 2015) while PCR is more effective than minimum inhibitory concentration (MIC) assays for detecting carbendazim resistance, it has certain limitations that reduce its practical utility. The method requires rapid thermal cycling, can suffer from insufficient specificity, and often exhibits low amplification efficiency, which can hinder consistent and reliable detection of resistant strains (Duan *et al.* 2016a; Duan *et al.* 2018b). Loop-mediated isothermal amplification (LAMP), developed in 2000, is a nucleic acid amplification technique designed for the rapid detection of pathogenic microorganisms. It operates under constant temperature conditions and offers high efficiency, specificity, and sensitivity, allowing precise and reliable amplification without the need for thermal cycling (Duan *et al.* 2018b; Fu *et al.* 2011; Notomi *et al.* 2000). LAMP is a gene amplification technique that operates at a constant temperature using a single enzyme. It is highly specific, efficient, sensitive, and rapid, making it a powerful tool for the detection of target nucleic acids without the need for complex thermal cycling (Duan *et al.* 2016a).

LAMP has been successfully applied for the detection of plant-pathogenic organisms because of its efficiency, simplicity, high specificity, and rapid amplification. Using a set of 4–6 carefully designed primers, nucleic acids can be rapidly amplified under isothermal conditions. This approach allows targeted DNA amplification in a single step without the need for advanced laboratory equipment, making it practical for field or resource-limited settings (Mori and Notomi 2009; Notomi *et al.* 2000). (Duan *et al.* 2016b) reported the efficiency of LAMP for the detection of F167Y and F200Y mutant genotype of F. asiaticum to carbendazim of which is used in detecting and monitoring MBC resistant problems and for the control of fusarium head blight in wheat field. LAMP has been effectively utilized to detect single-nucleotide mutations linked to fungicide resistance in fungi (Duan *et al.* 2014; Duan *et al.* 2016a; Duan *et al.* 2016b).

A LAMP assay was also developed for the simultaneous detection of multiple benzimidazole-resistant β-tubulin variants in *Botrytis cinerea* populations, enabling efficient monitoring of resistance under laboratory conditions (Duan *et al.* 2018b). One of the advantages of LAMP is its visual readout: highly benzimidazole-resistant isolates of *Sclerotinia sclerotiorum* were detected using a colorimetric change with hydroxynaphthol blue, allowing amplification results to be observed directly with the naked eye (Duan *et al.* 2015). LAMP was also used to detect the F200Y mutant genotype in carbendazim-resistant isolates of *Sclerotinia sclerotiorum* (Duan *et al.*, 2016b) and proved suitable for in-field detection of moderately carbendazim-resistant isolates of *Botrytis cinerea* in tomatoes, cucumbers, and strawberries (Duan *et al.*, 2018a).

Table 3: comparison of LAMP, PCR and MIC for detecting the highly carbendazim resistant populations of *S. sclerotiorum* from different fields of Jiangsu province of China in 2013

		LAMP		PCR		MIC	
Geographical origin	Number of samples	Positive	Resistance frequency (%)	Positive	Resistance frequency (%)	Positive	Resistance frequency (%)
Taizhou	387	76	19.64	72	18.6	78	20.16
Yancheng	117	23	19.66	24	20.51	23	19.66
Yangzhou	103	14	13.59	12	11.65	15	14.56
Suzhou	89	17	19.1	15	16.85	18	20.22
Huaian	104	25	24.04	24	23.08	25	24.04
Zhenjiang	64	15	23.44	13	20.31	15	23.44
Total	864	170	19.68	160	18.52	174	20.14

Source: (Duan *et al.* 2015).

CONCLUSION AND RECOMMENDATIONS

Benzimidazole fungicides are still in use for controlling plant pathogenic fungi, and therefore require close monitoring for resistance across a wide area to ensure effective and efficient disease management. The rapid development of pathogen resistance to benzimidazoles has prompted studies on their mode of action and resistance mechanisms. These fungicides were initially highly effective in managing plant diseases; however, the emergence of resistant strains poses a significant challenge. Benzimidazoles act by selectively binding with high affinity to pathogen β-tubulin, inhibiting microtubule polymerization, disrupting cellular structure, and ultimately causing pathogen death. Resistance to benzimidazoles arises from mutations affecting tubulin polymerization, a key component of microtubules. Combining benzimidazoles with fungicides from different chemical classes is an effective strategy for managing resistance. Understanding these mechanisms not only clarifies the fundamental mode of action and resistance processes in fungi but also supports monitoring, prediction, regulation, and large-scale management of resistance, while guiding the development of new fungicide formulations.

Author contributions

C. C. designed the study and supervised the experiments, J. I. M. performed the experiment, came up with the research idea and objectives, D. S. revised and edited, K. I. U. improved the paper, while K.P.B. prepare figures. All these authors contributed substantially to the final manuscript and approved this submission. All authors are aware of the authorship order and that no further change in authorship will be performed after submission.

Acknowledgements

This work was supported by Chinese key R & D Plan (2016YED0201007, 2018YFD0201201 and 2018YFD0201000), Agricultural Science and Technology Projects of Jiangsu Province, China [PZCZ201715 and CX (18)2005], Postgraduate Research &Practice Innovation Program of Jiangsu Province（No. KYCX18_0670).

Ethical Statement

This review was written in accordance with ethical guidelines. Informed consent was obtained from all authors, confidentially was maintained and no harm was caused

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