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# Molecular techniques for the improvement of microbial biocontrol agents against plant pathogens

Anik Majumdar[1*]

## Abstract

Biological control is a sustainable and ecologically effective method for bringing down pest population to an acceptable level. Implementing microbial biocontrol agents (MBCAs) to manage plant diseases necessitates the use of environmentally friendly practices that can increase global food production and guarantee the safety of food supply. Recent advancements in biotechnology have made it easier to find and characterize new beneficial microbes as well as to identify their genetic byproducts. These findings have made it possible to clone these microbes in plants in an effort to strengthen their resistance to biotic and abiotic stresses. The technological improvements have strengthened the symbiotic interaction between microbes and plants while also enabling the modification of the processes through which MBCAs exert their effects, ultimately enhancing their potential in managing plant diseases. The genome sequencing of MBCAs has yielded useful information about their genomes, which has helped to characterize them for efficiently. This article offers a thorough summary of the already existing and recent molecular advances used to increase the efficiency of MBCAs for managing plant diseases as well as to understand their biocontrol mechanisms through various omics technologies. These approaches are important for assuring food security and increasing agricultural outputs by minimizing yield loss due to plant diseases.

**Keywords** Microbial biocontrol agents, Genome sequencing, Omics, Advanced techniques

## Background

Research on the use of beneficial microbes for managing plant pathogens has been going on for more than 70 years, and it has started to show promise as a viable alternative to chemical control. Despite the fact that chemical control strategies are still frequently used to manage plant diseases, their potential future use may be constrained by the emergence of pesticide-resistant strains, the deregistration of pesticides and public worries about the potential negative effects of agrochemicals on human health and the environment. The reduction of pathogen populations by the use of living organisms is known as "biocontrol" of plant diseases (Heimpel and Mills 2017). Understanding the processes of biocontrol has long been the focus of research in microbiology, microscopy and biochemistry. The discovery of novel methods that improve our comprehension of the antagonistic processes used by MBCAs has recently been made possible by the development of molecular techniques, relying on the knowledge obtained through microbiological, microscopic and biochemical research. Furthermore, a deeper understanding of biocontrol techniques is being made possible by the expanding area of knowledge gained via genome sequencing (Abdel-Salarn et al. 2007). The study of biocontrol techniques, improving biocontrol activity, and discovering genes and metabolites that have major effects can all be studied by researchers using modern techniques of microbial genetics and molecular

*Correspondence:
Anik Majumdar
anikmajumdariari@gmail.com
[1] Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India

methods, which is going to be the major focus in this review. This review was designed with a clear objective: to compile all the existing as well as recent developments made for improving the efficacy of MBCAs in a single article. Since there are not many resources available describing the same in a concise way, this review may provide a unique opportunity to access the progress made over the years in the area of molecular techniques (from protoplast fusion to omics!) for improving the efficacy and understanding the biocontrol mechanisms of MBCAs, which indeed paves the way for managing plant diseases in a sustainable manner.

## Advanced techniques used for improving the efficacy of MBCAs

### Protoplast fusion techniques

Researchers have used protoplast fusion techniques to combine desired traits from other strains in order to improve the biocontrol abilities of MBCAs. In various *Trichoderma* strains, protoplast isolation, fusion and regeneration have been successfully accomplished (Brunner et al. 2005). The production of fungal cell wall-degrading enzymes (chitinase, endoglucanases) is one of the major weapons of *Trichoderma* spp. in their biocontrol mechanisms. *Trichoderma* HF9 fusant was generated by protoplast fusion between *Trichoderma harzianum* and *T. viride* (Balasubramanian et al. 2012). This strain was significantly more active than the parent strains in terms of both total and specific chitinase activity. Similarly, fusant FU3, derived from protoplast fusion between endophytic *T. harzianum* strains, showed improved antagonistic activity against *Aspergillus flavus, Sclerotinia sclerotiorum* and *Rhizoctonia solani* (Dolatabad et al. 2019). Ghasemi et al. (2019) reported that *T. harzianum* gamma radiation mutants T.h M8 and T.h M15 showed higher chitinase and endoglucanase production (as compared to the wild type) to inhibit the growth of *Rhizoctonia solani*. Similarly, to increase their antagonistic activity against soilborne pathogens like *R. solani, Sclerotium rolfsii, Fusarium oxysporum* and *Macrophomina phaseolina*, which cause root rot and wilt diseases, protoplast fusion was performed between *T. hazianum* NBAII Th 1 and *T. viride* NBAII Tv 23 (Lakhani et al. 2016). They were successful in producing fusants with enhanced antagonistic capabilities. In a different study, *Trichoderma* interspecific protoplast fusants were also developed to provide multi-stress tolerance (Hirpara et al. 2019). They found that, twenty of the 36 fusants had characteristics inherited from either parent, whereas 14 were heterozygous, displaying characteristics inherited from both parents. Notably, the fusant Fu21 showed biocontrol potential for stem rot disease. Improved MBCAs have also been used for the management of plant pathogenic nematodes. In order to increase the nematicidal potential of *Bacillus amyloliquefaciens* and *Lysinibacillus sphaericus* against *Meloidogyne incognita*, protoplast fusion approaches were studied by Abdel-Salam et al. (2018). Their results showed that protoplast fusion might be successfully used to enhance these bacteria's nematicidal properties. In another study, the performance of fusants derived from parent *Bacillus cereus* and *Pseudomonas aeruginosa* was evaluated against root knot nematode and weed seed germination. Fusants F7, F20 and F35 showed more antagonistic capabilities against *Meloidogyne incognita* J2 than parent strains. Moreover, they completely inhibited the germination of weed species *Portulaca oleracea* and *Echinochloa crus-galli* (Mohamed et al. 2021). Recently, mycoviruses have also been used to attenuate the virulence of plant pathogenic fungi. Hypoviruses can be introduced into virulent fungal strain from hypovirulent strain through hyphal anastomosis, provided they are vegetatively compatible. Lee et al. (2011) through protoplast fusion technique, introduced hypovirus FgV11-DK21 dsRNA into *Cryphonectria parasitica* and other *Fusarium* spp, resulting in reduced virulence of these pathogens. Yang et al. (2021) via protoplast fusion, introduced Cryphonectria hypovirus 1 (CHV1) and Mycoreovirus 1 (MyRV1) into apple valsa canker pathogen *Valsa mali*. The results showed that CHV1 and MyRV1 infected the pathogen significantly, leading to reduced virulence. Collectively, these results show that protoplast fusion is a useful technique for increasing the biocontrol potential of MBCAs in a variety of species, including *Trichoderma* and bacterial strains.

### Improving MBCAs via directed mutagenesis

Through chemical treatment or exposure to ultraviolet (UV) radiation, physical and/or chemical mutagens can cause mutations in bacterial and fungal MBCAs. Specific base changes that induce mismatches can be introduced into the double-stranded DNA molecule using compounds like ethyl methane sulfonate (EMS) or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG). On the other hand, UV radiation encourages the production of dimers from adjacent pyrimidines, which causes the insertion of an incorrect base at the dimer site. Additionally, frameshifts, duplications, or deletions in the DNA sequence can be brought on by either type of mutagens. In order to develop novel biotypes with improved skills as MBCAs or producers of antifungal metabolites, several authors have used mutagenesis techniques employing physical mutagens like gamma irradiation or UV radiation as well as chemical mutagens. One such gene *tvk1* was cloned from *Trichoderma virens*, which codes for a mitogen-activated protein kinase. In the *tvk1* null mutants, its

function in terms of mycoparasitism, conidiation and biocontrol were examined. When exposed directly to the plant pathogen *Rhizoctonia solani*, these mutants showed a considerable increase in the expression of genes associated to mycoparasitism (Mukherjee et al. 2003). Additionally, compared to the wild-type strain, the null mutants showed increased protein production as shown by high amounts of lytic enzymes in the culture supernatant. Marzano et al. (2013) isolated strains of *T. harzianum* that exhibited significantly improved biological control activity following UV mutagenesis. Similar to this, Abbasi et al. (2014) generated *T. harzianum* mutants with the highest growth inhibition against *Macrophomina phaseolina* using gamma rays. According to the study, melon plants treated with *Trichoderma* mutants had a 28% lower incidence of charcoal rot disease than the control. In order to improve the antagonistic properties of *T. harzianum*-1432 and *T. atroviride* against *Sclerotium rolfsii*, the causative agent of chickpea collar rot, NTG as a mutagen was used by Singh et al. (2016). Additionally, chemical mutagenesis has been used to increase the effectiveness of bacterial MBCAs. For instance, *Pseudomonas aurantiaca* B-162's nitrosoguanidine mutagenesis led to the identification of a strain with increased biocontrol activity and three times greater phenazine synthesis (Feklistova and Maksimova 2008). Yadav et al. (2023) used chemical mutagenic agents, NTG and nitrous acid ($HNO_2$) to improve the biocontrol efficacy of *Bacillus amyloliquefaciens* DSBA-11 against tomato bacterial wilt caused by *Ralstonia solanacearum*. The $MHNO_2$ strain, derived by treating *B. amyloliquefaciens* with $HNO_2$, was found to be more effective in controlling *R. solanacearum*. In another study, mutant strains of *Bacillus velezensis*, derived through UV radiation and EMS treatment, showed improved biocontrol efficacy. Mutant strain $M_3$ caused the highest antagonistic activity against *Helminthosporium maydis* (causal agent of southern corn leaf blight in maize) and also showed increased biosurfactant production (Rahman et al. 2023). Similarly, Manikandan et al. (2022) reported gamma-induced mutants of *Bacillus* spp. and *Streptomyces* spp. effectively antagonized the pulse root rot and wilt pathogen (*Macrophomina phaseolina* & *Fusarium oxysporum* f. sp. *udum*), respectively. It is worth noting that the genetic techniques employed in these studies do not involve recombinant DNA and merely simulate natural processes. Consequently, they do not fall under the regulations governing the use of genetically modified organisms, making them more readily acceptable for disease management. Mutation induction was also done using various EMS concentrations in an effort to produce improved strains of *Trichoderma viride*. According to the percentage of growth inhibition, the resultant *T. viride* mutants were efficient antagonists against *F. oxysporum* f.sp. *ciceris* (Dhandale et al. 2017). Similar to this, *Trichoderma harzianum* was exposed to gamma radiation using a Co-60 gamma radiator to cause mutations that improved antagonistic efficiency. Similarly, increased antagonistic activity against *Fusarium oxysporum* f.sp. *radicis-cucumerinum* was seen in the mutant strain *T. harzianum* CS5 (Sahampoor et al. 2020). Genetic enhancement of *Trichoderma asperellum* strains for improved efficacy as well as carbendazim tolerance were performed via NTG-induced mutations (Naik et al. 2023). Among the mutants obtained, N2-1, N2-2, N2-3 and N1 showed the highest antagonistic activity against *F. oxysporum* f.sp. *ciceri*, *Rhizoctonia bataticola* and *Botrytis cinerea*. Mutants N2-1, N2-2 and N2-3 also succeeded in sporulating even at 2000 µg/ml of carbendazim, thus proving their ability to tolerate high fungicide concentrations.

#### Genetic recombination and transformation

It is possible to increase the biocontrol activity of a MBCA by integrating foreign DNA into its genome that precisely codes for the biocontrol metabolites. In some strains, inhibiting or removing genes from the biocontrol strain can enhance long-term biocontrol action. These methods have been successfully applied to the management of *Agrobacterium tumefaciens* mediated crown gall disease, where a modified strain of *A. radiobacter* K84 was used to prevent the emergence of agrocin 84-resistant *Agrobacterium tumefaciens* strains through the transfer of plasmid genes (de Boer et al. 2003). In another study, the genomic DNA of *Trichoderma viride* included a gene that codes for endochitinase. This DNA fragment, spanning 1672 base pairs, contains a promoter and coding region for mRNA. The gene was introduced to *Chaetomium globosum* CG10 after being combined with the *Aspergillus nidulans Trp synthetase* promoter and terminator sequences. About one-third of the transformed strains were observed to have a considerable increase in endochitinase activity (Schnider et al. 1995). A *chiA* gene encoding the major chitinase of *Serratia marcescens* was inserted into an endophytic strain of *Pseudomonas fluorescens* (isolated from micropropagated apple plantlets) which effectively controlled *Rhizoctonia solani* on bean seedlings in a plant growth chamber (Downing and Thomson 2000). It was found that *P. fluorescens* Rif1 strain expressing the *chiA* gene caused improved chitinase activity, which led to the suppression of the pathogen on beans. Additionally, the competitive colonization ability of both the poor and good colonizer strains of *P. fluorescens* on tomato root tips was greatly improved by the insertion of the *sss* colonization gene from the *F. oxysporum* f. sp. *radicis-lycopersici* biocontrol strain. This suggests that genetic engineering can

realistically improve colonization ability (Dekkers et al. 2000). Recently, Zhang et al. (2020) developed recombinant *Pseudomonas synxantha* strain ZHW 15 and ZHW 25, which exhibited improved biocontrol activity against several wheat pathogens. The recombinant strains were developed by introduction of pyrrolnitrin coding genes from *Pseudomonas protegens* Pf-5 to *P. synxantha* 2-79. Both of the recombinant strains showed improved biocontrol efficacy compared to the parent strain.

Mycoparasitism, a mode of action employed by antagonists, involves the degradation of pathogen cell walls through the production of lytic enzymes such as chitinases, proteases and glucanases. Genes encoding chitinases have been successfully cloned from various microorganisms, including *Serratia marcescens* (Sundheim et al. 1988), and *T. harzianum*. *T. virens* cotton strains with two copies of the *ech42* gene and a defective *ech42* gene fragment were shown to have enhanced and decreased antagonistic activity against *Rhizoctonia solani* respectively (Baek et al. 1999). On agar plates, constitutive expression of *chit33* in *T. harzianum* caused enhanced biocontrol activity against *R. solani* (Limón et al. 1999). Likewise, the endochitinase-encoding gene *Chi67-1* was introduced into *Clonostachys rosea* to increase its biological activity against *Sclerotinia* rot of soybean (Sun et al. 2017).

#### Transposon mutagenesis

A number of mutants have been produced by the random insertion of transposable elements such as Tn5 or Tn3 into the genome of an organism to improve its biocontrol activities (Simon et al. 1983). For instance, 2,4-diacetylphloroglucinol (2,4-DAPG) biosynthetic locus *phlACBDE*, which was cloned from *Pseudomonas* strain CPF-10, was successfully assembled into a mini-Tn5 transposon (Zhou et al. 2005). Then, this construct was inserted into the chromosome of the non-2,4-DAPG-producing strain *Pseudomonas fluorescens* P32. The resultant strains considerably increased resistance to bacterial wilt induced by *Ralstonia solanacearum* in tomato as well as resistance to take-all disease in wheat caused by *Gaumannomyces graminis* var. *tritici*. Likewise, a group of random transposon (Tn) mutants from *Pseudomonas parafulva* JBCS1880 were employed in a different study by Kakembo and Lee (2019). These mutants showed high antagonistic activity toward *Xanthomonas axonopodis* pv. *glycines* and *Burkholderia glumae*. This was the first time a *P. parafulva* strain, which is anticipated to produce a novel lipopeptide, was used to control bacterial pustules on soybeans. Additionally, Alsohim (2020) investigated on the effects of *P. fluorescens* mutants generated through transposon mutagenesis. Their research showed that these mutations efficiently reduced *Rhizoctonia solani*, *Alternaria* sp. and *Colletotrichum* sp. mycelial development, consequently enhancing the potency of the bacterial isolates to stimulate plant growth. In a recent study conducted by Luo et al. (2023), surfactin (a bioactive compound with antimicrobial properties) production was found to be increased by *B. velezensis* Bs916 through transposon mutagenesis. In this study, 20 derivatives with high surfactin production were selected via transposon mutagenesis. The derivative H5, mutated for *GltB* gene (δ*GltB*), was found to produce seven times more surfactin than the wild type.

#### Cloning and insertion of genes responsible for antibiotic/antifungal enzymes, siderophore and bacteriocin production

To expand the range of biologically controlled pathogens, it is possible to transfer genes responsible for antibiotic biosynthesis from one antagonistic strain to another. For example, a genetically modified strain of *Pseudomonas putida* has been developed to enhance its activity against soilborne pathogens. According to Bakker et al. (2002), this transformed strain produced phenazine-1-carboxylate (PCA) or 2,4-diacetylphloroglucinol (DAPG) on a constitutive basis and contained the *phz* or *phl* biosynthetic gene loci. In another work, trichodermin was produced more abundantly after the trichodermin gene *(tri5-trichodiene synthase*) was cloned and expressed in *T. brevicompactum*. Trichodermin exhibits antifungal properties that are effective against *Aspergillus fumigatus* and *Fusarium* spp. (Ma et al. 2003).

Extracellular enzymes including chitinases, cellulases and β-1,3-glucanases, which break down the components of plant pathogenic fungal cell walls, are produced by both bacterial and fungal MBCAs. These lytic enzymes are often encoded by single genes, facilitating their isolation and transfer to other MBCAs. For example, when tested on agar plates, recombinant strains of *T. harzianum* transformed with the *chit33* gene showed improved biocontrol activity against *R. solani* (Reino et al. 2008). In a study by Ellatif et al. (2022), genes encoding xylanase enzyme was cloned from *T. harzianum* kj81197. The xylanase-producing gene *xyn2* exhibited largest xylanase activity compared to the wild type and showed superior antifungal activity against *Alternaria* spp., *F. oxysporum*, *Corynespora cassiicola* and *Botrytis cinerea*. Similarly, *ChiA* gene encoding chitinase was derived from *Bacillus velezensis* and was cloned into *E.coli*. The resulting recombinant protein, rBVChiA exhibited improved antifungal activity against black pepper pathogen *Fusarium falciforme* (Tran et al. 2022). Additionally, PCA-producing biocontrol strains like *P. fluorescens* and *P. aureofaciens* were able to produce phenazine-1-carboxamide (PCN) instead of PCA due to the transfer of

the *phzH* gene from *Pseudomonas chlororaphis*, which is necessary for PCN biosynthesis and control of *F. oxysporum* f. sp. *radicis-lycopersici*. As a result, *P. fluorescens* and *P. aureofaciens* effectively suppressed tomato foot and root rot diseases through PCN production (Chin-A-Woeng et al. 2001).

Siderophore production is one of the many mechanisms through which MBCAs outcompete the pathogens for nutrients uptake. MBCAs may chelate iron with greater specificity and affinity using compounds called siderophores. Iron sequestration by siderophores reduces the amount of this crucial nutrient available to other microbes, which inhibits their development. Molecular approaches have been extensively used to study the role of siderophore synthesis by rhizosphere bacteria in biological control against phytopathogens. The use of heterologous siderophores can provide bacteria a competitive edge in the rhizosphere. For instance, *Pseudomonas* sp. B24, a fluorescent strain of *Pseudomonas*, was genetically altered to improve its capacity to use more ferric siderophores (Moënne-Loccoz et al. 1996). The plasmid pCUP2 containing the *pbuA* gene, which encodes the membrane receptor of ferric pseudobactin M114, was introduced into the engineered *Pseudomonas* strain, enabling it to utilize both its own siderophore and the pseudobactin siderophore produced by *P. fluorescens* M114. This genetic alteration expanded the siderophore production that the modified *Pseudomonas* strain could utilize, possibly giving it an advantage in the rhizosphere environment. Recently, siderophore-producing genes have been identified in *Lysobacter enzymogenes*, which encode the novel siderophore lysochelin (Miller et al. 2023). Similarly, siderophore-producing genes have been identified in *Bacillus subtilis* YB-04 through genome analysis (Xu et al. 2022). These genes can be cloned and transferred into other MBCAs for improving their siderophore-producing capabilities, ultimately leading to improved efficacy against plant pathogens.

Crown gall disease in pome and stone fruits is known to be caused by the soil bacteria *Agrobacterium tumefaciens*. Agrocin 84, a bacteriocin produced by its non-pathogenic counterpart *Agrobacterium radiobacter* strain K84, is essential for the biocontrol mechanism of crown gall disease (Kerr 1980). The plasmid pAgK84 contains the genes for agrocin 84 synthesis, bacteriocin immunity and conjugal transfer (*tra*). Through the use of recombinant DNA technology, a novel strain of *A. radiobacter* was produced in order to increase the effectiveness of biological management against crown gall disease. *A. radiobacter* strain K1026 was the result of the deletion of a 5.9 kb area that overlapped the transfer (*tra*) region of plasmid pAgK84 (Jones et al. 1988). This genetic modification was aimed to reduce the risk of breakdown in the biological control of crown gall. Currently, crown gall on stone fruits is being managed commercially using the genetically engineered strain K1026. Studies have demonstrated that it is equally effective to the parent strain K84 in terms of root colonization as well as managing the crown gall disease (Jones and Kerr 1989). Recently, a novel bacteriocin, Lcn972 was found in *B. velezensis* HN-Q-8 and expressed in *E.coli* to derive the bacteriocin. The bacteriocin (which is also stable against high UV radiation) caused satisfactory inhibition of the potato scab pathogen *Streptomyces scabies* (Zhao et al. 2022). Similarly, Wang et al. (2020) characterized a novel bacteriocin, carocin S3 (which also includes the killer protein carocin S3K and the immunity protein carocin S3I), from multiple bacteriocin-producing strains of *Pectobacterium caratovorum* subsp. *caratovorum*.

#### Gene identification techniques

The isolation and sequencing are the first step in the characterization of any gene. There are two types of gene identification strategies: targeted and open. The targeted approach focuses on precisely identifying one or a small number of relevant genes. The open strategy, on the other hand, is used to find more genes by methods like differential expression analysis, extensive sequencing, or random mutation. In the early molecular research on biocontrol mechanisms, the focus was primarily on genes encoding enzymes that directly impacted plant pathogens. Examples of such enzymes include glucanases, proteases and chitinases, which continue to make up a sizeable fraction of the identified genes. Later, researchers turned their focus to the genes that control the actions of the aforementioned enzymes. Thus far, the identification of genes in MBCAs has predominantly been limited to the *Trichoderma* genus, with only a few genes identified in other genera.

In the targeted strategy, the initial step involves designing degenerate primers that amplify specific segments of the gene sequence. These primers are carefully chosen based on factors such as the amino acid sequence of the protein being studied, sequence alignment of similar proteins from different microorganisms, or previous primer used for other MBCAs. Once the degenerate primers are in place, the DNA fragment is amplified through polymerized chain reaction (PCR) and subsequently isolated, cloned and sequenced. For instance, *T. harzianum* endo-1,3-glucanase (Donzelli et al. 2001) genes and *T. harzianum* exo-1,3-glucanase gene (Ait-Lahsen et al. 2001) have been successfully isolated using this method. Using degenerate primers that targeted conserved regions found by multiple amino acid alignment of known exoglucanase genes, Grevesse (2002) identified exoglucanase activity in culture filtrates. They effectively amplified

exo-1,3-glucanase genes in post-harvest MBCAs, particularly *Pichia anomala*.

In the open strategy, one effective approach involves the utilization of techniques that examine differential gene expression. These techniques have been extensively employed in studies focused on identifying genes associated with biocontrol. Notably, methods such as cDNA amplified fragment length polymorphism analysis (cDNA-AFLP), differential display and subtractive hybridization have been utilized thoroughly. cDNA-AFLP was used in a study to identify genes that may be involved in the biological control of *Botrytis cinerea* by *Pichia anomala* strain Kh5 (Massart and Jijakli 2006). In a medium containing either glucose or the cell wall components of *Botrytis cinerea*, the strain Kh5 was cultured. By using this method, they were able to identify eleven cDNA fragments that were related to the genes overexpressed in the presence of *B. cinere*a cell wall components. These fragments were thought to have a possible role in the secretion of enzymes. Another study conducted by Liu and Yang (2005) focused on the sequencing of *T. harzianum* cDNAs using the expressed sequence tags (ESTs) technique. In this investigation, they found 55 cDNAs that had similar characteristics with proteins related to biocontrol. These cDNAs were further classified according to their possible modes of action, which included pathogen protease inactivation (5 cDNAs), mycoparasitism (22 cDNAs), competition for resources and space (5 cDNAs) and antifungal activity (23 cDNAs).

#### Biocontrol-related promoters

To achieve successful transformation and demonstrate biocontrol activity, it is necessary to identify appropriate promoters related to biocontrol activities. A modified version of the naturally occurring bacteria *Erwinia ananas* (NR1), which is present in rice, was developed in a study. The altered strain of the bacterium, as opposed to the original strain, contained genes from *Serratia marcescens* B2, particularly those that encoded antifungal components such as the 58-kDa endochitinase. These antifungal factor genes expression was regulated by promoters derived from the recipient *E. ananas*. The result of the study showed that the rice blast disease could be resisted by the genetically altered microbe. Therefore, using the host's own promoter to express foreign genes is a novel strategy to achieve expression of biocontrol associated genes (Wu et al. 2011).

#### Improved root colonizing abilities

Certain soil bacteria exhibit beneficial effects on plants without establishing a symbiotic relationship. These bacteria, referred to as plant growth-promoting rhizobacteria (PGPR), are widely present in the soil, around the roots and even inside plant roots. By preventing the establishment of plant pathogenic bacteria, PGPR boost plant growth in a significant way. Researchers have frequently assessed the possibilities of PGPR genetic modification to improve their capacity for biocontrol of phytopathogens. Enhancing the ability of PGPR strains to compete for limited nutritional resources including carbon, nitrogen and iron is one of the methods for increasing the ability of these strains to colonize plant roots. Manipulating genes associated with signaling pathways involved in colonization, such as those governing motility, chemotaxis and biofilm formation, can also enhance the colonization of biocontrol bacteria. Barahona et al. (2011), reported a hypermotile mutant strain of *P. fluorescens* that was developed by disrupting the *kinB, sadB* and *wspR* genes. This strain outperformed the wild-type strain in terms of rhizosphere colonization and demonstrated effective control over *F. oxysporum* and *Phytophthora cactorum*. The two-component signal transduction system *DegU-DegS* regulated flagellar motility and biofilm formation in *Bacillus* species, and the DegQ protein influenced these processes by increasing DegU phosphorylation. Xu et al. (2019) developed *B. velezensis* as a recombinant strain by substituting the *degQ* gene with a xylose-inducible *degQ*. They showed that xylose, a carbohydrate often produced by plant roots, stimulated biofilm formation by this recombinant strain. When compared to the wild-type strain, the recombinant strain greatly increased the portion of roots colonized by it on cucumber and tomato plants, and it was also more effective in preventing tomato bacterial wilt and cucumber *Fusarium* wilt diseases. In a recent study, Chi et al. (2023), functionally characterized glycosyltransferase Taugt17b1, which is known to enhance the root colonizing ability of *Trichoderma* species. They reported that the overexpression of *Taugt17b1* increased *Trichoderma atroviridae* colony growth rates, improved its root colonizing ability and induced systemic defense responses in plants to prevent plant diseases.

### Genome sequencing of MBCAs

Whole-genome sequencing has identified distinctive traits of several microbes with the potential to be developed as MBCAs. Using paired-end and whole-genome shotgun sequencing methods, Takeuchi et al. (2014) completed a comprehensive genome sequencing of the rhizospheric bacteria *Pseudomonas* sp. Cab57. Using gap-spanning PCR products, they were able to bridge the gaps between the contigs. They discovered four gene clusters (*phl, prn, plt* and *hcn*) that govern the generation of antibiotics, nitrite/nitrate absorption and the Gac/Rsm signal transduction pathway. Additionally,

it was discovered that these genes improved the bacterium's capacity to inhibit the growth of pathogenic microbes. In a different work, Jiang et al. (2017) reported the whole-genome sequence of the naturally occurring MBCA *Bacillus cereus* AR156 that exhibits potential in managing soilborne diseases. The capacity of this MBCA to protect tomato plants from *Meloidogyne incognita*, a nematode that causes root knot disease, as well as *Ralstonia solanacearum*, the cause of bacterial wilt, was demonstrated. Piombo et al. (2018) demonstrated the genome sequencing, assembly and characterization of two yeast cells belonging to the species *Metschnikowia fructicola*. These yeast cells demonstrated great biocontrol efficiency against post-harvest infections that lead to produce spoiling in fruits and vegetables. The release of cell wall-degrading enzymes, stimulation of defense signaling genes, presence of iron-binding compounds and a wide generation of superoxide anions were identified by the researchers as the biocontrol activities of the yeast strain. Few of the potential microbial biocontrol agents whose genome have been sequenced recently are enlisted in Table 1.

## Genomics

The term "omics" refers to a broad area that includes a number of scientific disciplines, including proteomics, metabolomics, peptidomics, transcriptomics, phenomics, interactomics, genomics and secretomics (Sharma et al. 2017). The metabolome and expressome of various MBCAs, including *Trichoderma*, have been identified and analyzed using these highly effective omics techniques. The importance of omics in understanding the complex and varied biotic interactions involved in MBCAs like *Trichoderma* was emphasized by Lorito et al. (2010). The complete study of genomes is called genomics and it is a subfield of omics. It has made a significant contribution to our comprehension and advancement about biological control. It is now feasible to limit pathogen activity and create new consortia of MBCAs by understanding their microbiomes. The identification of multiple genes present in *Trichoderma* biocontrol strains has benefited

**Table 1** Recent complete genome sequences of potential microbial biocontrol agents (published in and after 2015)

| S. no. | Microbial biocontrol agents | Accession numbers | References |
|---|---|---|---|
| 1 | *Trichoderma virens* FT-333 | JTGJ00000000 | Kuo et al. (2015) |
| 2 | *Trichoderma harzianum* T6776 | JOKZ00000000 | Baroncelli et al. (2015) |
| 3 | *Trichoderma gamsii* T6085 | JPDN00000000 | Baroncelli et al. (2016) |
| 4 | *T. harzianum* B97 | MRYK00000000 | Compant et al. (2017) |
| 5 | *Trichoderma* sp. ITEM908 | PNRQ00000000 | Fanelli et al. (2018) |
| 6 | *Trichoderma afroharzianum* T11-W and *T. cyanodichotomus* TW21990-1 | WUWT00000000 and WXUD00000000 | Zhou et al. (2020) |
| 7 | *Trichoderma reesei* QM6a | PRJNA225530 | Martinez et al. (2008) |
| 8 | *Trichoderma parareesei* CBS125925 | LFMI00000000 | Yang et al. (2015) |
| 9 | *Trichoderma hamatum* GD12 | ANCB00000000 | Studholme et al. (2013) |
| 10 | *T. virens* Gv29-8 | PRJNA264113 | Kubicek et al. (2011) |
| 11 | *Bacillus cereus* UW85 | LYVD01000000 | Lozano et al. (2016) |
| 12 | *Bacillus atrophaeus* GQJK17 | CP022653 | Ma et al. (2018) |
| 13 | *B. velezensis* UFLA258 | NZ_CP039297 | Olishevska et al. (2019) |
| 14 | *B. velezensis* PG12 | PIWI000000000 | Zeng et al. (2019) |
| 15 | *B. velezensis* AL7 | CP045926 | Liu et al. (2020) |
| 16 | *B. velezensis* YB-130 | CP054562 | Xu et al. (2020) |
| 17 | *Pseudomonas fluorescens* BRZ63 | PRJNA529642 | Chlebek et al. (2020) |
| 18 | *Bacillus subtilis* WS1A | JABFHE000000000 | Rahman et al. (2020) |
| 19 | *B. subtilis* MBI600 | CP033205.1 | Samaras et al. (2021) |
| 20 | *B. subtilis BS87* | SRR11870891 | Chandra et al. (2021) |
| 21 | *B. subtilis BBv57* | SRR17459383 | Thiruvengadam et al. (2022) |
| 22 | *B. subtilis* PTA-271 | JACERQ000000000 | Leal et al. (2021) |
| 23 | *B. velezensis* AK-0 | CP047119 | Kim et al. (2021) |
| 24 | *Bacillus amyloliquefaciens* TA-1 | JARDRQ000000000 | Wang et al. (2023a) |
| 25 | *B. amyloliquefaciens* Bam1 | CP082279 | Luo et al. (2022) |
| 26 | *B. amyloliquefaciens* Cas02 | CP071932 and CP071933 | Chu et al. (2022) |

greatly from genomic data. A genome mining strategy has also discovered the novel orphan biosynthetic cluster genes and made it easier to find related secondary metabolites in biocontrol strains (Eyiwumi et al. 2015). Similarly, genome sequencing and annotated gene function studies of important bacterial MBCAs have yielded useful information regarding their biocontrol mechanisms. For example, eight gene clusters with the potential for antimicrobial secondary metabolite production were identified from *B. atrophaeus* GQJK17 (Ma et al. 2018). PacBio sequencing of *B. velezensis* YB-130 revealed 12 gene clusters responsible for secondary metabolite production, with special mention to lanthipeptide (Xu et al. 2020). Similarly, *B. subtilis* MBI600, with a genome size of 40,76,736 bp, exhibited higher root colonizing ability and antagonistic activity against both *Pythium aphanidermatum* and *F. oxysporum* f.sp. *radicis cucumerinum* in cucumber (Samaras et al. 2021). Thiruvengadam et al. (2022) conducted complete genome sequencing of *B. subtilis* Bbv57 (genome size of 4,302,465 bp), an important MBCA with nine gene clusters involved in the production of antimicrobial secondary metabolites. Additionally, Wang et al. (2023a, b) reported that *Bacillus amyloliquefaciens* TA-1 has a single circular chromosome with 4172 protein-coding genes. Through in vitro assays, they demonstrated that the TA-1 strain is a potent antagonist against the groundnut early leaf spot pathogen, *Cercospora arachidicola*.

#### Proteomics

Proteomics is essential for understanding how the host, pathogens and microbial biocontrol agents interact with each other. It allows for the identification of new proteins that can be isolated and used to combat plant diseases. In early days, proteomic investigations pertaining to biocontrol activities were mostly conducted using gel-based techniques. However, the accessibility of gel-free mass spectrometry-based techniques has increased the viability of proteomic research of MBCAs. In comparison to older gel-based approaches, these cutting-edge methodologies have greatly enhanced protein analysis studies (Otto et al. 2012). Liquid chromatography and tandem mass spectrometry (MS) analyses have widespread applications in studying MBCAs characteristics and to learn more about the MBCA proteomic fingerprints and its underlying biological processes. Proteomic techniques may be used to identify proteins that are involved in biocontrol processes, have biotechnological potential and have differential expression (Alfonso et al. 2016). For instance, *Trichoderma* proteomic analysis discovered the presence of fungal cell wall-degrading enzymes (CWDEs), including three novel proteins produced by *T. atroviride* in response to the plant pathogen *R. solani*: *N*-acetyl-b-D-glucosaminidase and endochitinase (Grinyer et al. 2005). *T. harzianum* stimulated the synthesis of various CWDEs, including b-1,6-glucanases, proteases, chitinases and xylanases in the growing medium when it interacted with the plant pathogen *B. cinerea* (Yang et al. 2009). Furthermore, proteomics can help in identifying the development of immunity in plants treated with MBCAs like *Trichoderma*. A study demonstrated the identification of numerous positive and negative protein effectors in the interaction between maize roots and *T. virens*. These effectors play a role in suppressing defense pathways and inducing systemic resistance (Lamdan et al. 2015).

In another context, studying the three-way interaction between the host, pathogen and MBCAs could enhance our understanding of MBCA-mediated defense responses in host plants. A proteomic study, involving two-dimensional gel electrophoresis–matrix-assisted laser desorption ionization time-of-flight mass spectrometry (2DE-MALDI-TOF-MS) by Prabhukartikeyan et al. (2019) showed that tripartite interactions between rice–*Bipolaris oryzae*–*Bacillus amyloliquefaciens* led to the expression of proteins involved in plant metabolism and defense responses. Similarly, through 2DE-MALDI-TOF analysis in the three-way interaction between rice–*Rhizoctonia solani*–*Bacillus subtilis* EPB24, upregulation of putative disease resistance proteins such as serine–threonine protein kinases, β-1,3-glucanases, nucleotide-binding site–leucine-rich repeats (NBS-LRR) proteins and peroxidases was observed (Durgadevi et al. 2021). Paranthaman et al. (2023), through 2DE-MALDI-TOF analysis, reported that tomato roots inoculated with *P. fluorescens* Pf1 showed upregulation of defense-related proteins such as phenylalanine ammonia lyase, serine–threonine protein kinase and nucleoside diphosphate kinase, which eventually contributed to host defense against *F. oxysporum* f.sp. *lycopersici*. Proteomic studies could also provide valuable information regarding the pathogen's response to a particular MBCA, which is crucial for formulating effective management strategies. For example, acetyl-proteomic analysis of the apple valsa canker pathogen, *Cytospora mali*, revealed its lysine acyetylome in response to *B. velezensis* L-1 inoculation. Although *B. velezensis* L-1 could reduce the virulence of the pathogen, *Cytospora mali*, in turn, could alleviate MBCA suppression through the upregulation of acetylated proteins in autophagy, cytochrome P450 and heat-shock protein 70 (Sun et al. 2022).

#### Transcriptomics

Transcriptome studies offer an excellent approach for characterizing candidate transcripts involved in various biological functions. ESTs, subtractive libraries

and cDNA micro-arrays are some of the tools that may be used to analyze the vast transcriptomic responses to various factors and relate it to certain biological processes (Herrera-Estrella 2014). On studying the relationships between *Trichoderma* spp., plants and pathogens, several transcriptome research have been conducted. For instance, Reithner et al. (2011) examined *T. atroviride* IMI206040 transcriptome at various phases of interaction with *R. solani*. *Phytophthora capsici* and *B. cinerea*. Through quantitative reverse transcription PCR, it was found that thirteen genes showed differential transcription (in the presence of *R. solani*), which mostly code for expansin-like proteins, acetyl xylan esterases, aspartyl proteases and trypsin-like proteases. Moreover, when relative gene expression analysis of these genes was studied at different stages of parasitism against *B. cinerea* and *P. capsici*, it revealed a synergistic transcription of several genes responsible for fungal cell wall degradation. Wang et al. (2023a, b) conducted a study on mycoparasitism-associated genes of *T. harzianum* T4 using transcriptomic analysis. The *T. harzianum* T4 strain effectively controlled the tomato gray mold pathogen *B. cinerea*. Approximately 2871 differentially expressed genes (DEGs) were identified in *T. harzianum* T4 at 12, 24, 48 and 72 h of growth in the presence of the pathogen's cell wall. The top ten significantly upregulated genes across all time periods encoded for glycoside hydrolases, cutinases, proteases, secondary metabolite synthesis, signal transduction proteins, etc. Additionally, Zhao et al. (2020) conducted a comprehensive analysis of the genome and transcriptome of *Coniothyrium minitans*, a mycoparasite that targets the plant pathogen *Sclerotinia sclerotiorum*. The research aimed to gain a better understanding of the molecular processes underlying the mycoparasitic relationship. Transcriptomic profiling of bacterial MBCAs has also contributed deciphering their biocontrol strategies. In a recent study by Jiang et al. (2019), it was discovered that the plant growth-promoting strain *B. velezensis* F21 is an effective biocontrol agent capable of suppressing the development of *Fusarium niveum* in watermelon while enhancing resistance and immunity in the plant. Transcriptomic analysis revealed that nearly a thousand ripening-related genes were upregulated in watermelon roots treated with *B. velezensis* F21 and *Fusarium niveum*, demonstrating the establishment of systemic resistance in response to the infection. Another transcriptomic study involved tomato roots pre-treated with MBCA *Paenibacillus polymyxa* NSY50, which resulted in the upregulation of defense-related genes such as pathogenesis-related (PR) proteins and phenylalanine ammonia lyase. In plants inoculated with NSY50 and the *Fusarium* wilt pathogen, 390 DEGs were identified, with 294 genes upregulated and 96 genes downregulated (Du et al. 2022). Mu et al. (2023) reported the response of the tomato early blight pathogen *Alternaria solani* to the MBCA *B. amyloliquefaciens* XJ5 through transcriptomic analysis. They found that after MBCA inoculation, a total 174 DEGs were observed, including 60 upregulated genes and 114 downregulated genes. At 24 and 48 h post-inoculation with XJ5 crude protein extracts, there was significant downregulation of chitin and mannose synthesis genes in *A. solani*, potentially impacting its ability to synthesize cell walls and resulting in reduced growth.

### Secretomics

Secretomics is a specific branch of proteomics that focuses on the analysis of secreted proteins. This analytical method is very useful for detecting the whole protein repertoire generated by MBCAs, such as *Trichoderma*. It is a well-established fact that different *Trichoderma* species colonize host plants and protect them from pathogen infections. In a study conducted by da Silva et al. (2022), secretomes were derived from culture filtrates following the growth of *T. harzianum* TR 274 in the presence or absence of *Phaseolus vulgaris*. Approximately 124 and 190 fungal proteins were identified in the presence and absence of the host, respectively. The main functional proteins identified were glycoside hydrolases, proteases and oxidoreductases, primarily responsible for modulating host defense responses. Secretome analyses have also been done to decipher the biocontrol mechanisms of MBCAs. For instance, the mycoparasitic activity of *T. harzianum* ALL42 is reported to be associated with a number of important proteins, including chitinases, glucoamylases, β-1,3-glucanases and proteases. After 24 h of incubation, these proteins were found to be activated when the cell wall of *Fusarium solani* was introduced to the culture medium (Ramada et al. 2016). Additionally, it was shown that *T. harzianum* secreted a large number of proteins during antagonistic interactions with the plant pathogen *Guignardia citricarpa*. These secreted proteins were responsible for inducing plant defense mechanisms and inhibiting the growth of the pathogen mycelium (de Lima et al. 2017). In a similar study, Rueda-Mejia et al. (2021) conducted a secretome analysis of *Aureobasidium pullulans* isolate NBB 7.2.1. in the presence of *F. oxysporum*. Among 10,925 annotated genes, 79 genes were detected at the protein level, which were predicted to be associated with glycosylases, esterases, proteases, as well as secondary metabolite synthesis. These antagonistic proteins eventually caused reduced growth of *F. oxysporum* in the media.

## Conclusions and future directions

As stated above, this review highlights the various molecular techniques that have been applied to improve the efficacy of MBCAs and understand their biocontrol mechanisms to combat plant pathogens. However, there are still much to learn about the mechanisms underlying advantageous plant–microbe interactions. Despite the market potential, a number of issues including limitations in fermentation, formulation, registration and commercialization need to be solved. The developments in next-generation technologies have offered great information on the interactions between plants and beneficial microbes. Characterizing these MBCAs requires a thorough understanding of their genomes. So, to gain a comprehensive understanding of the biocontrol strains, it is preferable to first identify their properties using genomic, transcriptomic and proteomic approaches. This background information can help in finding pathways that are important in the biocontrol mechanisms. Recently, omics methods have provided opportunities to understand the mechanisms that are used by MBCAs, while genetic manipulation through molecular techniques has the potential to develop new biocontrol strains with improved tolerance to biotic stresses and increased production of toxic compounds toward plant pathogens. However, it is important to note that genetic manipulation of MBCAs is still in its early stages, and regulatory considerations significantly impact its acceptance. Global attention has been drawn to the rising necessity to monitor genetically modified organisms under different environmental conditions. The growing body of knowledge in biotechnology can aid in the development of methods for monitoring the genetic and environmental effects of genetically modified MBCAs, thus enabling their long-term usage in the management of plant diseases.

**Abbreviations**

| | |
|---|---|
| MBCAs | Microbial biocontrol agents |
| UV | Ultraviolet |
| EMS | Ethyl methane sulfonate |
| NTG | *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine |
| 2,4-DAPG | 2,4-Diacetylphloroglucinol |
| PCA | Phenazine-1-carboxylate |
| PCN | Phenazine-1-carboxamide |
| PCR | Polymerase chain reaction |
| cDNA-AFLP | CDNA amplified fragment length polymorphism |
| CWDEs | Cell wall-degrading enzymes |
| ESTs | Expressed sequence tags |
| 2DE-MALDI-TOF-MS | Two-dimensional gel electrophoresis–matrix-assisted laser desorption ionization time-of-flight mass spectrometry |
| NBS-LRR | Nucleotide-binding site–leucine-rich repeats |
| DEGs | Differentially expressed genes |

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