



Review article

Fungal endophyte bioinoculants as a green alternative towards sustainable agriculture

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ABSTRACT

Over the past half century, limited use of synthetic fertilizers, pesticides, and conservation of the environment and natural resources have become the interdependent goals of sustainable agriculture. These practices support agriculture sustainability with less environmental and climatic impacts. Therefore, there is an upsurge in the need to introduce compatible booster methods for maximizing net production. The best straightforward strategy is to explore and utilize plant-associated beneficial microorganisms and their products. Bioinoculants are bioformulations consisting of selected microbial strains on a suitable carrier used in the enhancement of crop production. Fungal endophytes used as bioinoculants confer various benefits to the host, such as protection against pathogens by eliciting immune response, mineralization of essential nutrients, and promoting plant growth. Besides, they also produce various bioactive metabolites, phytohormones, and volatile organic compounds. To design various bioformulations, transdisciplinary approaches like genomics, transcriptomics, metabolomics, proteomics, and microbiome modulation strategies like gene editing and metabolic reconstruction have been explored. These studies will refine the existing knowledge on the diversity, phylogeny and beneficial traits of the microbes. This will also help in synthesizing microbial consortia by evaluating the role of structural and functional elements of communities in a controlled manner. The present review summarizes the beneficial aspects associated with fungal endophytes for capitalizing agricultural outputs, enlists various multi-omics techniques for understanding and modulating the mechanism involved in endophytism and the generation of new bioformulations for providing novel solutions for the enhancement of crop production.

1. Introduction

Booming global population, increased food security, environmental and climatic fluctuations, and a shortage of tillable land are few of the unprecedented hurdles in the envisioned sustainable agriculture goals [1]. This has resulted in the intensification of the crop yield per unit area of soil used, known as agricultural intensification which is the most efficient and prioritized method for meeting world's healthy food demands [2,3]. The conventional methods used for intensifying crop production are based on the excessive use of

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fertilizers and pesticides [4]. The uncontrolled and prolonged input of these agrochemicals adversely affects the ecosystem structure and function [5]. In view of this, microbes and microbial products have turned out to be the new frontier for fostering innovation, engineering, and the development of bioformulations for achieving ecological balance and sustainability. This has expedited the research on microbial diversity for revitalizing the green revolution by improving agricultural productivity [6].

The endophytic microbial community, in particular, has undergone millions of years of coevolution within the host plant and resulted in the generation of responsive feedback in host physiology [7]. The plant and its endobiome have been considered a mini-ecosystem in which the endobiome acts as an essential determinant for the overall growth and development of the plant. They either colonize intercellularly or intracellularly in the healthy and living tissues of host plants to complete the whole or part of their life cycle. The association with the host plant ranges from symbiotic to pathogenic, however, they are mostly in a symbiotic relationship. The host provides living space and nutrition to the endophytes, and in return, they bestow the plant with various advantages like triggering an immune response and imparting tolerance by producing secondary metabolites, proteins, and hormones [8].

The putative attributes of fungal endophytes can be suitably translated and implemented as bioinoculants, biostimulants, biofertilizers, and biopesticides. These efficacious microbial bioproducts circumvent the hazardous outcomes associated with conventional farming practices [9]. Furthermore, the spectacular progress in molecular biology, synthetic biology, high-throughput screening, enzyme discovery, protein engineering, metabolic engineering, and multiomics approaches has provided insights into the mechanistic understanding of various metabolic networks for delivering agronomic solutions [10].

Therefore, the present review provides an understanding of the beneficial attributes of plant-associated fungal endophytes, their molecular mechanisms, and modern sequencing technologies for identifying and shaping the endophytic community. Besides, various fungal bioformulations for enhancing agricultural productivity in a sustainable manner have also been discussed.

2. Fungal endophytes as bioinoculants for sustainable agriculture

Fungal endophytes are a polyphyletic group of predominantly ascomycetous fungi with *Fusarium*, *Aspergillus*, *Alternaria*, *Trichoderma*, *Penicillium*, *Cladopsorium*, *Colletotrichum*, and *Talaromyces* species being the most prevalent [11,12]. They have been explored mostly for their ability to produce a plethora of bioactive secondary metabolites, such as alkaloids, phenolics, quinones, steroids, saponins, tannins, and terpenoids. These metabolites are reservoirs of novel and potent sources of antimicrobial, anti-insect, anti-cancer, antioxidative, and antifungal compounds, among many other properties of biotechnological interest [13,14]. They are intended to facilitate plant health by improving soil structure and encouraging the host to overcome various biotic and abiotic stresses.

The detailed advantages presented by fungal endophytes are discussed below.

2.1. Stress management

Biotic factors such as phytopathogens and abiotic stresses such as drought, cold, heat, salinity, and variations in the pH of soil majorly affect the agricultural output. Further, varying climate conditions and human activities also add to the already existing challenging conditions [15]. To overcome this situation, the exploitation of useful microbial flora is the most reliable option [16].

2.1.1. Biotic stress management

Plant diseases are responsible for major crop losses at the global level [17]. Endophytes play a major role in the biocontrol of phytopathogens by producing various compounds such as phytoalexins and PR-proteins and also by stimulating the thickening of the cell wall and cuticle that helps the plant show a defense response [18]. They show antagonistic activity against phytopathogens by producing antimicrobial, antioxidant, antitumor, and insecticidal metabolites [19]. The most commonly used fungal biocontrol agents are *Trichoderma harzianum*, *Trichoderma viride*, and mycorrhizal fungi. Amongst them, the use of mycorrhizal fungi as a biocontrol agent has gained immense importance and has been known to protect plants against soil-borne pathogens such as *Pythium*, *Fusarium*, *Macrophomina*, etc. [20]. *Trichoderma* sp. are also well known for their ability to induce resistance in host plants, which makes them a popular biocontrol agent for plant pathogens [21,22].

Bian et al. [23] described *Epicoccum dendrobii* as a potent suppressive agent of *Colletotrichum gloeosporioides*. Kapoor et al. [24] examined the antimicrobial activity of fungal endophytes, *Porostereum* sp. and *Aspergillus* sp., isolated from *Dysoxylum gotadhora* (Buch. Ham.) Mabb., against *Fusarium oxysporum* and *Verticillium dahlia*. Positive results were witnessed, signifying their importance in providing resistance responses to the plants. Fungal endophytes have also been widely reported to protect plants from all types of insect pests including aphids, lepidopterous larvae, and thrips [25]. *Beauveria bassiana* and *Metarhizium anisopliae* are among the extensively studied entomopathogenic fungi. Advanced research studies and commercialization of endophytes as biocontrol agents are important to pave the way towards sustainable agriculture [26].

2.1.2. Molecular mechanism of plant endophyte interaction during biotic stress

Understanding the molecular effects of endophytic colonization on the host surface and interior during pathogen contact is critical. The plant-endophytic interaction during a pathogen attack causes a change in second messengers such as Ca^{2+} in the cytosol, which functions as a signalling molecule in the detection of microbe-associated molecular patterns (MAMPs) and triggers the complicated immune response. Pattern recognition receptors (PRRs) on the surface of plant cells recognise MAMPs. This early stage of defense induction is known as MAMP-triggered immunity (MTI) [27]. In addition to MTI, during a pathogen attack, the plant immune system is triggered by tiny secreted proteins known as elicitors via direct or indirect recognition through a sequence of plant resistance (R) gene products to suppress the MTI. Plant R genes, the majority of which are nucleotide-binding leucine-rich repeat (NB-LRR) domain

receptors (NLRs), recognise certain pathogen effectors, also known as avirulence or AVR proteins, and activate effector-triggered immunity (ETI). ETI is distinguished by a hypersensitive reaction (HR), which results in localised cell death [28].

Resistance is often induced by several phytohormones, such as jasmonic acid (JA), ethylene (ET), and salicylic acid (SA). The JA and ET routes promote resistance against necrotrophic diseases, and the SA pathway promotes resistance against biotrophic and hemibiotrophic pathogens [29]. Endophytic colonization of roots and the expression of pathogenesis-related genes are frequently associated with the elicitation of induced systemic resistance to infection. Endophytes have been shown to generate a variety of lytic enzymes, including cellulose, chitinase, hemicelluloses, proteases, and amylases, which help in the hydrolysis of polymers [30]. Lytic enzymes are important in the colonization of endophytes in host cells by forming polysaccharides and protein biofilms. They also aid in the management of plant diseases via the cell wall disintegration process [31].

Endophytes confer resistance to the host by competing with pathogens for nutrition, space, and resources in nutrient-rich environments due to niche overlap [32]. Endophytes also produce a variety of metabolites in plants, including steroids, alkaloids, phenolics, flavonoids, and terpenoids, which have a role in innate immunity and defensive response signalling [33]. A fungal endophyte, *Phomopsis* sp., produces VOCs that contain butanol, acetone, sabinene, 1-butanol, and phenethyl alcohol, that hinder the development of ascomycetes and deuteromycetes [34]. Several antioxidant enzymes, such as peroxidase (POD), phenylalanine ammonia lyase (PAL), and superoxide dismutase (SOD), aid in the defense against oxidative stress and lipid peroxidation during pathogen invasion. Enzymes such as catalases, peroxidases, superoxide dismutases, glutathione-S transferases, and alkyl hydroperoxide reductases neutralise ROS that is damaging to plants [35].

Various strategies used by fungal endophytes in promoting defense reactions in plants are outlined in Fig. 1 [36].

2.1.3. Abiotic stress management

2.1.3.1. Role of fungal endophytes in improving drought and salinity stress tolerance in plants. Among various abiotic factors, drought and salinity are the main stress conditions responsible for the decrease in total crop yields [37,38]. They have a severe influence on the overall physiology of plants and have a deep impact on nutrient uptake and photosynthetic activity. Around 20% of the total arable

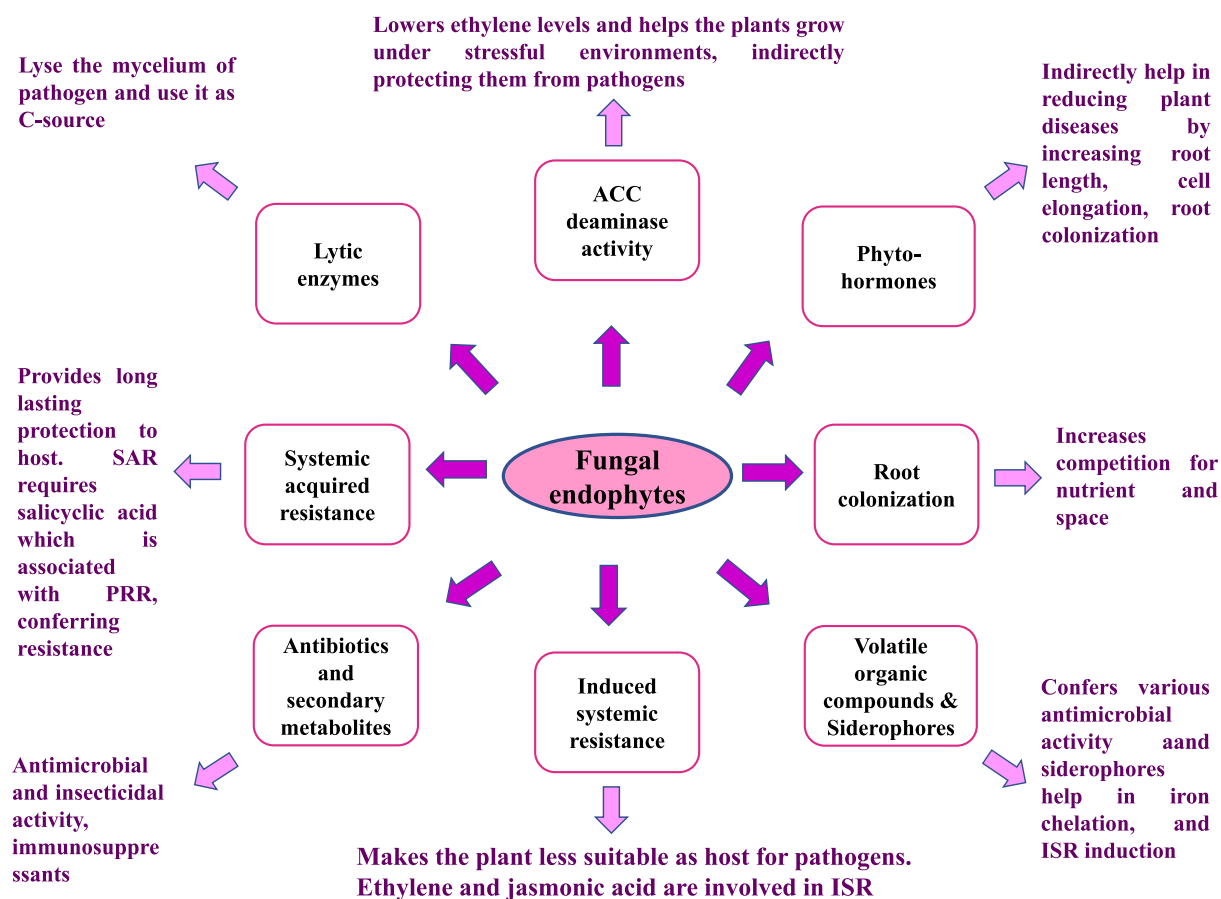


Fig. 1. Defense strategies used by fungal endophytes against phytopathogens.

land faces salinity stress, which is estimated to extend up to 30% by 2050. However, the effect depends on the intensity and period of exposure. In addition to this, less rainfall and increasing temperatures play an important role in escalating the salt concentration. To cope with adverse environmental conditions, it is important to develop stress tolerance in plants. The plant growth promotion activity of endophytes and their ability to produce natural compounds can be explored to induce stress tolerance in plants, and increase crop productivity [39–41]. They play a major role in acclimatizing host plants under abiotic stress situations by stimulating stress-responsive genes. Exogenous application of endophytes stimulates the adaptive behaviour of the host plants by modifying their genome, proteome, epigenome, and metabolome. Plants inoculated with endophytes have been observed to possess higher tolerance levels against drought and salinity stress as compared to non-inoculated ones [15]. In addition to this, they have been shown to accumulate more solutes and have a thicker cuticle than non-inoculated plants, which helps them survive drought stress [40].

Zhou et al. [42] witnessed enhanced seedling growth in *Pinus tabulaeformis* after inoculating it with *Phoma* sp. under drought conditions. Recently, Heosseyini et al. [43] explored the effect of three halotolerant endophytic fungi isolated from *Zygophyllum eichwaldii*, *Seidlitzia rosmarinus*, and *Haloxylon ammodendron*, found in the central desert of Iran, on salinity and drought stress tolerance in two model plants, cucumber and tomato. Agronomic parameters such as chlorophyll content, nitrogen uptake, and other physiological markers like proline content and the activities of various enzymes were assessed. The fungal endophytes were found to increase chlorophyll concentration, proline content, antioxidant enzyme actions, and overall plant growth.

2.1.3.2. Endophyte mediated molecular mechanism during drought and salinity stress. Abiotic stress increases ROS production, which creates an oxidative stress environment in the cell resulting in protein denaturation, nucleotide disruption, and lipid peroxidation. This may affect the plant's physiology leading to its death. However, in equilibrium conditions, plants maintain a sheer balance between the production and quenching of reactive oxygen species but their metabolic state gets affected above certain tolerance levels. Generally, plants produce several enzymes in response to high concentrations of ROS. In addition to enzyme production, activation of non-enzymatic systems also takes place in plants that involves quenching of ROS [44]. The loss of this equilibrium between the production and quenching of ROS is responsible for the deteriorating state of crop plants.

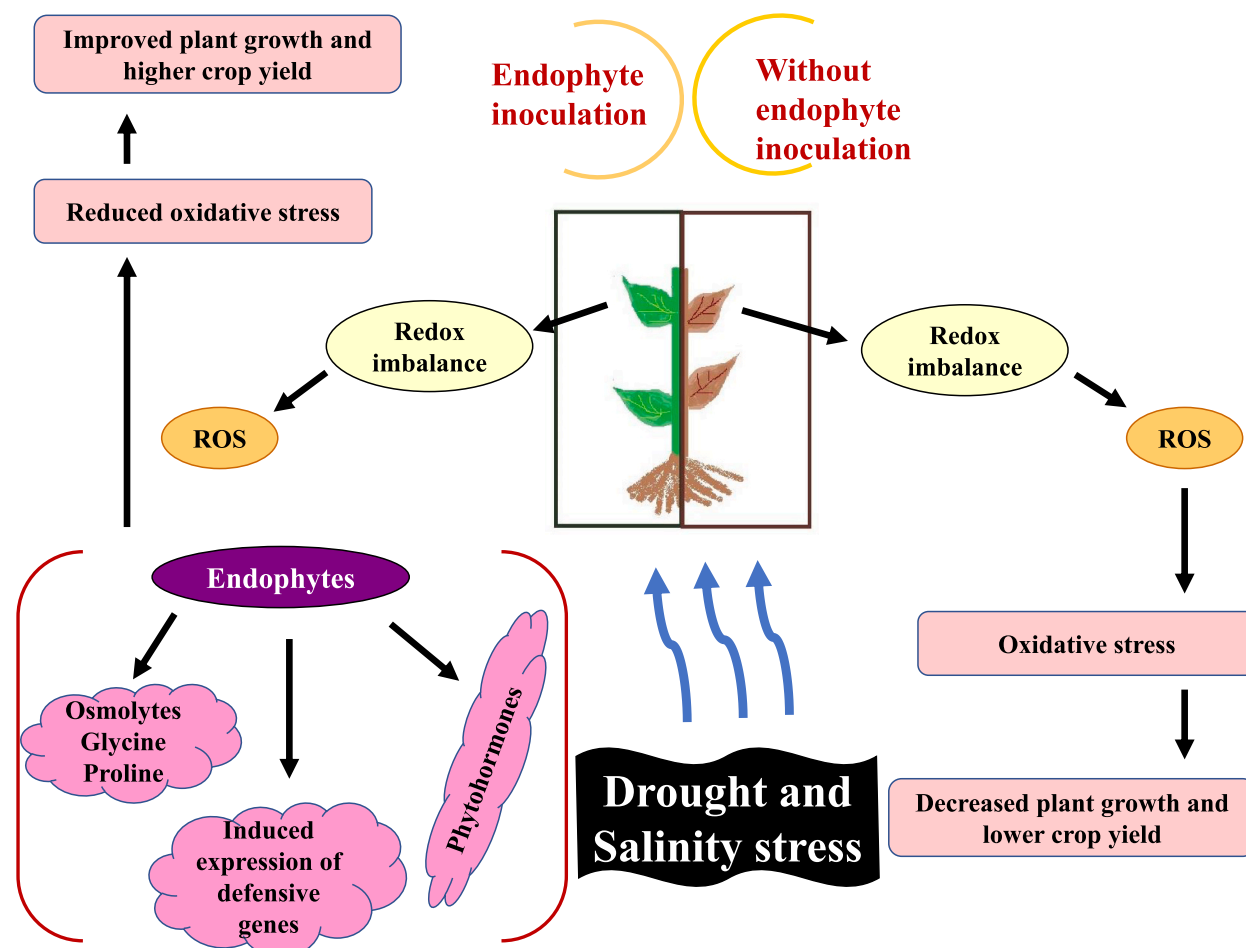


Fig. 2. Endophyte mediated molecular mechanism during drought and salinity stress.

The inoculation of endophytes into the plant notably lessens the oxidative stress damage caused by abiotic stress factors by enhancing the levels of antioxidant enzymes such as superoxide mutase, catalase, and ascorbate peroxidase, as well as non-enzymatic antioxidant molecules like carotenoids, glutathione, and ascorbic acid [40]. This creates a balance of free radicals that maintains optimum cell functioning. The colonization of endophytes also regulates the expression levels of certain genes responsible for tolerating stressful environments. This has been demonstrated in a study that looked at the synergistic association between *Piriformospora indica*, an endosymbiont, and *Brassica campestris* subspecies *chinensis*. It has been noted that increasing the expression levels of specific salt tolerance genes, such as SOS1, SOS2, and *NHX*-type Na⁺/H⁺ antiporter (*NHX1*) genes, can lessen the negative effects of salinity stress. The levels of antioxidant enzymes and phytohormones, mainly GA and SA, which significantly increase stress tolerance, have also increased [45]. In addition to this, they also produce a range of osmolytes that maintain the sodium-potassium ratio, overcoming the osmotic effect caused by stressful situations [15]. The molecular mechanism underlying the abiotic stress response is illustrated in Fig. 2.

2.1.3.3. Role of fungal endophytes in improving heat and cold tolerance levels in plants. With the change in global climate conditions, the temperature is continuously fluctuating which affects the crops on a major scale. Low temperature decreases the photosynthetic rate and increases H₂O₂ accumulation in cells. High temperatures severely hamper mineral acquisition, development of pollen grains, and shoot growth, and also disrupt respiration and photosynthetic processes. In addition to this, a rise in temperature increases the accumulation of ROS and disrupts cell membrane fluidity. All these conditions adversely affect crop yield [46].

Zhou et al. [47] demonstrated that the exogenous inoculation of *Epichloe* endophyte with calcium nitrate stimulated root metabolic activity, leaf relative water content, and photosynthetic pigment accumulation, which could improve the survival of *Festuca sinensis* grass under cold stress. Additionally, Karimi et al. [48] examined the effects of the inoculation of a fungal endophyte, *Piriformospora indica*, in grapevine under cold stress conditions. The endophyte inoculation increased the concentration of osmoregulators, plant growth regulators, and activity of antioxidant enzymes to mitigate cold stress.

2.2. Nutrient acquisition

Acquiring insoluble nutrients from soils is a significant hurdle in the growth of terrestrial plants. Hence, they form a symbiotic association with beneficial microorganisms to acquire nutrients from the soil. Endophytes have been shown to help plants acquire various nutrients, such as nitrogen, phosphorus, potassium, and other minerals [49]. Various studies have been conducted to evaluate their role in mineral solubilization, especially in increasing nitrogen and phosphorus concentrations in the shoots and roots of plants [50].

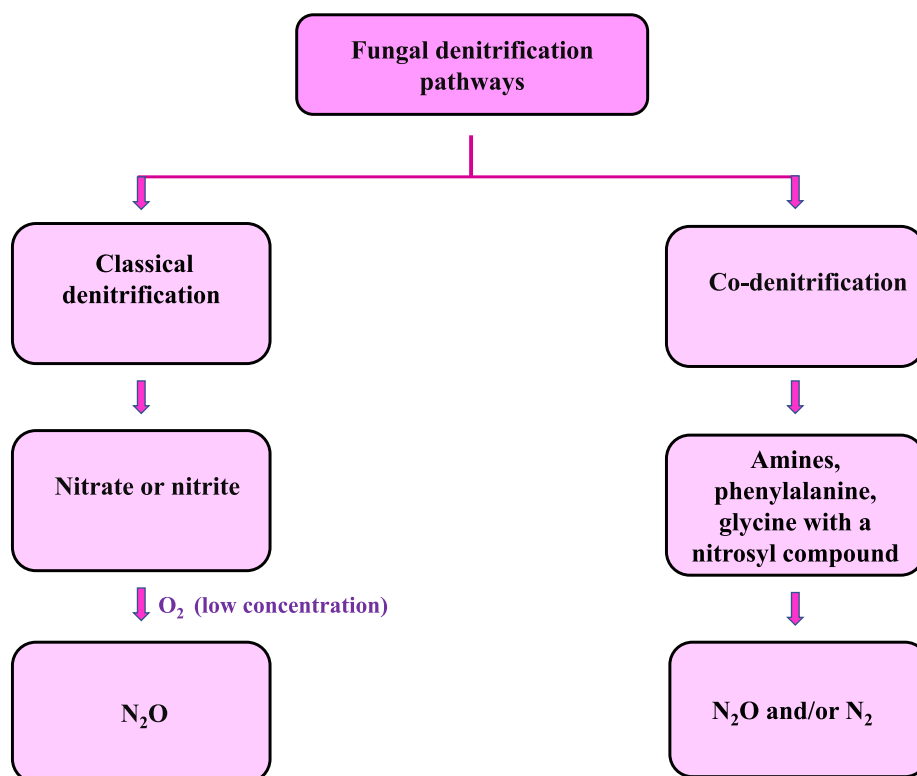


Fig. 3. Pathways involved in fungal denitrification process.

2.2.1. Nitrogen uptake by fungal endophytes

Microbes that live both inside the host tissues and outside in the soil form another kind of nutritional endophytic symbiosis. This type of symbiosis is established with numerous plant families by dark septate endophytes (DSE) and mycorrhizal fungi. Their hyphae develop endophytically in the roots, and their mycelia that extend into the soil gather nutrients and transport them back to the plants [51]. Vergara et al. [52] evaluated the impact of DSEs in the uptake of nitrogen and other nutrients in rice plants. The study suggested that the fungal endophyte, A103 improved nitrogen recovery from ammonium sulphate and increased the growth of rice plants by inducing the expression of H^+ -ATPase isoforms, Osa5 and Osa8 and stimulating the activity of PM H^+ -ATPase and H^+ -pyrophosphatase. Another method of acquiring nutrients is through the release of nutrients from insects by EIPF (Endophytic Insect Pathogenic Fungi), which infects and kills insects in the soil and directly transfers nitrogen obtained from insects to plants [53].

Endophytic fungi and nitrogen cycle: an underlying mechanism: The nitrogen cycle involves different steps, including denitrification, nitrogen fixation, nitrification, ammonium oxidation and ammonification. Arbuscular mycorrhizal fungi are not directly involved in nitrogen fixation, but instead, they increase the nitrogen fixing capacity of bacteria like *Rhizobia* and *Azospirillum*, which are present in the rhizosphere. They majorly play a role in denitrification and co-denitrification (Fig. 3). The main genes responsible for this are *nirK* (copper-containing nitrite reductase), *P450nor* (cytochrome P450 nitric oxide reductase), *dNar*, and *aNar* [54].

Various reports indicate the role of fungal endophytes in nitrogen acquisition. Yakti et al. [55] reported that the *Periconia macrospinos* increased tomato growth by increasing the nitrogen uptake from organic sources. Another study witnessed the rise in peanut yields following the inoculation of *Phomopsis liquidambaris*, resulting in enhanced nitrogen fixation and nodulation processes. The underlying mechanism suggests that the root exudates procured from the *P. liquidambaris* colonization reduced nitrate concentration while increasing *Bradyrhizobium* sp. population. This is due to improved interaction between the peanut and *Bradyrhizobium* sp. which improves nitrogen levels in peanut. The decrease in nitrate concentration increased the availability of other nutrients such as ammonium, DON (Dissolved Organic Nitrogen), DOC (Dissolved Organic Carbon), AK (Available Potassium), and AP (Available Phosphorus), that may promote plant growth [56].

2.2.2. Phosphate solubilization

Phosphorus is the second most important nutrient required for plant growth after nitrogen [57]. Its free availability is limited due to its structural and chemical properties and also due to the numerous fixation reactions taking place during the biogeochemical cycling of phosphorus. Hence, it is not easily utilised by plants. To decrease the usage of chemicals and meet the phosphorus demand, it is advisable to explore microflora that can solubilize the insoluble phosphorus present in the soil [58].

P-solubilizing (phosphorus solubilizing) fungal endophytes mostly belong to the genera *Aspergillus*, *Penicillium*, *Curvularia*, and *Piriformospora*, and endophytic arbuscular mycorrhizal (AM) fungi belong to the genera *Gigaspora*, *Sclerocystis*, *Acaulospora*, *Glomus*, *Entrophospora*, and *Scutellospora* [59]. Numerous studies have reported the potential of fungal endophytes for solubilizing phosphorus. Adhikari et al. [60] determined the phosphate solubilizing efficiency of five endophytic fungi isolated from the roots of *Taxus wall-ichiana*. All the isolates were found to solubilize phosphate by using substrates such as aluminium, iron, and calcium phosphate, along with the secretion of phytases and phosphatases. Similarly, Noorjahan et al. [61] screened fungal endophytes from seaweeds for their phosphorus solubilizing capability and observed that *Aspergillus* sp., *Penicillium oxalicum*, and *P. citrinum* possessed maximum activity.

Mechanism of P-solubilization employed by fungal endophytes: There are two types of P-solubilization mechanisms employed by fungal endophytes-inorganic and organic P-solubilization mechanisms, depending on the source present in the soil.

Inorganic P-solubilization: It involves the secretion of organic acids (acetic acid, gluconic acid, citric acid, maleic acid, glycolic acid, oxalic acid, succinic acid, lactic acid), inorganic acids (sulphuric acid, carbonic acid, nitric acid), siderophores, and exopolysaccharides. The most potent mechanism for inorganic P-solubilization is the release of organic acids. These acids are released from the outer surface of the cytoplasmic membrane and form a complex that chelates cations linked with phosphorus and releases soluble phosphorus into the soil through ligand exchange interactions with phosphate anions. Their efficacy depends on the number and position of hydroxyl and carboxyl groups, the type and quantity of organic acid and metal, the stability constant of the complex formed by metal and organic acid, and the pH of the soil solution [62].

Organic P-solubilization: It includes the discharge of diverse enzymes, phosphatases, phosphonates and phytases. Dephosphorylation or hydrolyzation of ester and phosphoanhydride bonds is done by phosphatases [57]. Around fifty percent of organic phosphate is present in the seeds and pollen of plants in the form of phytate. Phytases digest phytate and release the free available form of phosphate. In addition to this, the carbon-phosphorus bond of organophosphates is hydrolyzed by phosphonates [62].

2.2.3. Iron acquisition by siderophore production

Harsh environmental circumstances lead to less availability of iron due to the formation of insoluble oxyhydroxide phases. Iron deficiency in plants leads to chlorosis, less metabolic activity, and reduced biomass production. Hence, plants and their associated microbes have adopted a chelation approach for stimulating the availability of iron during stressful situations [63]. Different classes of siderophores, such as ferrichromes, coprogens, and fusarinines, are produced by fungal endophytes for iron acquisition [64]. Around 500 siderophores have been reported till date. The structure of siderophores produced by fungal endophytes is different from the ones produced by bacterial endophytes in that they have acylated ornithine groups rather than acylated alkylamines. In a study, Rajini et al. [65] observed siderophore production in 23% of fungal isolates procured from *Sorghum bicolor*. Similarly, Chowdhary et al. [66] evaluated the plant growth promotion potential of endophytic fungi isolated from the inflorescence of *Aloe vera* L. and observed that *Trichoderma harzianum* showed positive plant growth promoting traits, including siderophore production. Fungal endophytes also inhibit the growth of phytopathogens by reducing the amount of iron available to them, which further hinders the formation of nucleic

acids and the sporulation of plant pathogens. Hence, they contribute to the biocontrol properties of fungal endophytes.

Mechanism of action: The two oxygen molecules in the hydroxamate group of siderophores ($\text{C}(=\text{O})\text{N}-(\text{OH})-\text{R}$) serve as chelating agents, forming hexadentate octahedral complexes with Fe^{3+} with high stability constants [63]. The complex then binds to particular receptor proteins present on the microbial surface, gets translocated by an active transport system, and is released inside the cell [64].

2.3. Emission of volatile organic compounds

Modulation of plant defense responses by fungal endophytes is majorly attributed to the production of volatile organic compounds (VOCs). Around 322 secondary metabolites have been reported to date, and these compounds are derived from the metabolic pathways of fungal endophytes. VOCs are hydrophobic compounds with high vapour pressure and a low molecular weight. They include terpenoids, peptides, alkaloids, quinones, phenols, and xanthenes and play a vital role in plant-fungal communication. They can cross plant cell membranes due to their physiochemical properties and, hence, play a major role in maintaining the soil ecosystem [67].

The biosynthesis of VOCs majorly depends on the accessibility of nitrogen, carbon, sulphur, and energy emitted by primary metabolic pathways, namely the mevalonic acid (MVA), lipoxigenase (LOX), shikimate, and methylerythritol phosphate (MEP) pathways (Fig. 4). The precursors involved in these pathways are acetyl-CoA, E4P, PEP, and pyruvate.

VOCs secreted by fungal endophytes have a major impact on the growth of plants and in their defense mechanisms, and hence can be used as “green chemicals” [68].

2.4. Production and regulation of phytohormones

Phytohormones play a significant role in modifying plant physiology. There are reports on the involvement of fungal endophytes in regulating hormone levels in their hosts. The most vital hormone produced during stress conditions is abscisic acid (ABA), also known as the “plant stress hormone”. It activates the stress-responsive genes, thereby increasing the tolerance level in plants against various stresses like drought, heat, cold, etc. Other important phytohormones that help plants survive stress conditions are auxins, ethylene, gibberellic acid, etc. Sometimes adverse external environments severely decrease the phytohormone concentration which produces harmful effects on plant growth that may lead to plant death. Under these circumstances, fungal endophytes help elevate the hormone levels in plants by producing various hormones such as auxins, gibberellins, and cytokinins and also regulating their levels [69].

IAA (indole-3-acetic acid) is the majorly produced auxin by endophytic fungi. It has several positive effects on plant growth such as root and shoot development, cell elongation, the initiation of the process of root formation, and many others. The main precursor involved in IAA synthesis is L-tryptophan, but the whole metabolic pathway is not yet defined. Another important hormone produced by fungal endophytes is “gibberellins”. They are involved in various plant processes such as seed germination, sexual expression, fruit formation, stem elongation, and senescence. Gibberellins are produced through the mevalonic acid (MVA) pathway with acetyl-CoA as a precursor. The final products formed are GA_1 and GA_3 [70]. Two hormones that are involved in induced systemic resistance are ethylene and jasmonic acid. On the other hand, salicylic acid has a major role in the systemic acquired resistance mechanism. The interplay between these three hormones is necessary for producing defense reactions in plants [71,72].

Saad et al. [73] suggested that the plant growth promoting and antagonistic activity of *Nigrospora sphaerica*, a fungal endophyte isolated from *Melia azedarach*, might be attributed to its ability to produce IAA hormone in tomatoes. Wu et al. [74] found that the alteration in plant hormone metabolic pathways by a dark septate fungal endophyte (isolated from *Anteaglonium* sp.) promoted

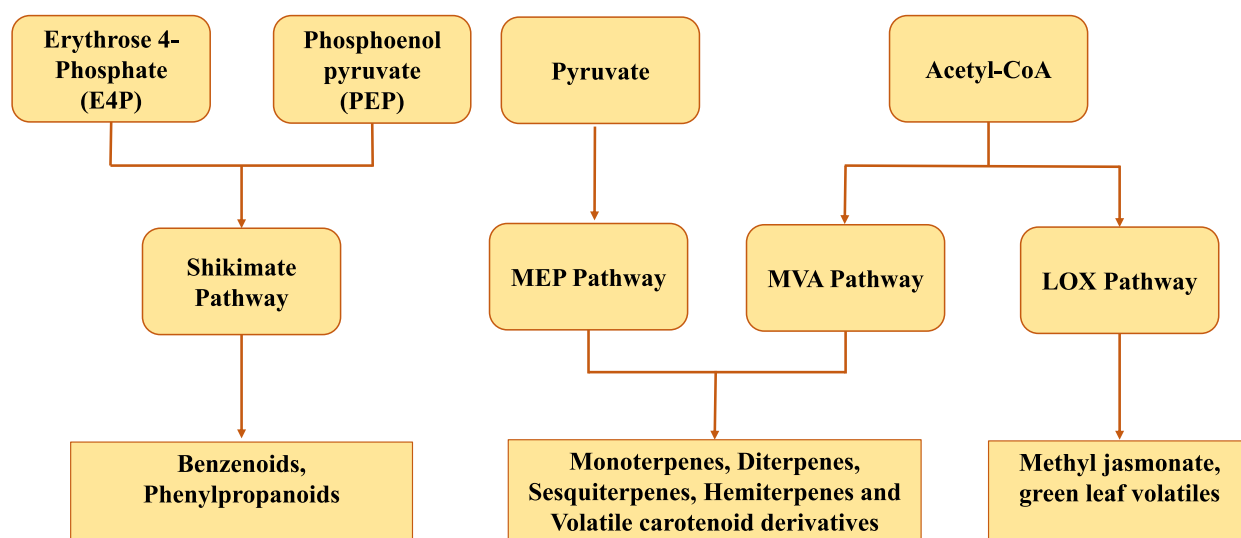


Fig. 4. Biosynthetic pathway of Volatile Organic Compounds.

blueberry growth. Hence, the ability of fungal endophytes to produce phytohormones makes them suitable for use as biostimulants.

3. Modern biotechnological approaches for shaping microbial bioinoculants

To achieve enhanced productivity and sustainable agriculture, it is crucial to understand the interaction and mechanism of action of fungal bioinoculants. New methods are constantly evolving with the use of novel interdisciplinary approaches like sequencing technology and systems biology [75]. The systems biology approach is based on the design-build-test-learn (DBTL) cycle, which involves inoculating plants with plant growth promoting microbes and monitoring interactions, generating datasets, integrating them, identifying candidate genes, and finally evaluating their function through gene editing to determine future approaches and improvements [76]. Various approaches to emphasizing the omics tools for the development and shaping of microbial bioinoculants are discussed below (Fig. 5).

3.1. Genomics approach

Genomics is a branch of biology that focuses on determining the structure, function, evolution, mapping, and editing of genomes through high-throughput genome sequencing and bioinformatic analyses. Most of the conventional studies have involved the genetic techniques of a single mutation to detect the resultant phenotype and have even led to the discovery of many genes. Metagenomics allows for the study of all microorganisms, cultivated or not, by analyzing genetic data taken directly from an environmental sample, providing knowledge of the species present as well as information on the functionality of microbial communities in their natural habitat. With great success, functional metagenomics has been used to find numerous new genes, proteins, and secondary metabolites, such as antibiotics, with industrial, biotechnological, pharmacological, and medicinal applications. The comparative genomics strategy has also been used to improve knowledge of plant growth promotion and symbiosis by simplifying the comparative examination of high-quality accessible data on symbiotic microbes in public repositories. The functional genomics approach has been fundamental in the cataloguing of gene function.

A comparative genomics investigation of three endophytic *Aspergillus* species indicated the existence of additional carbohydrate-

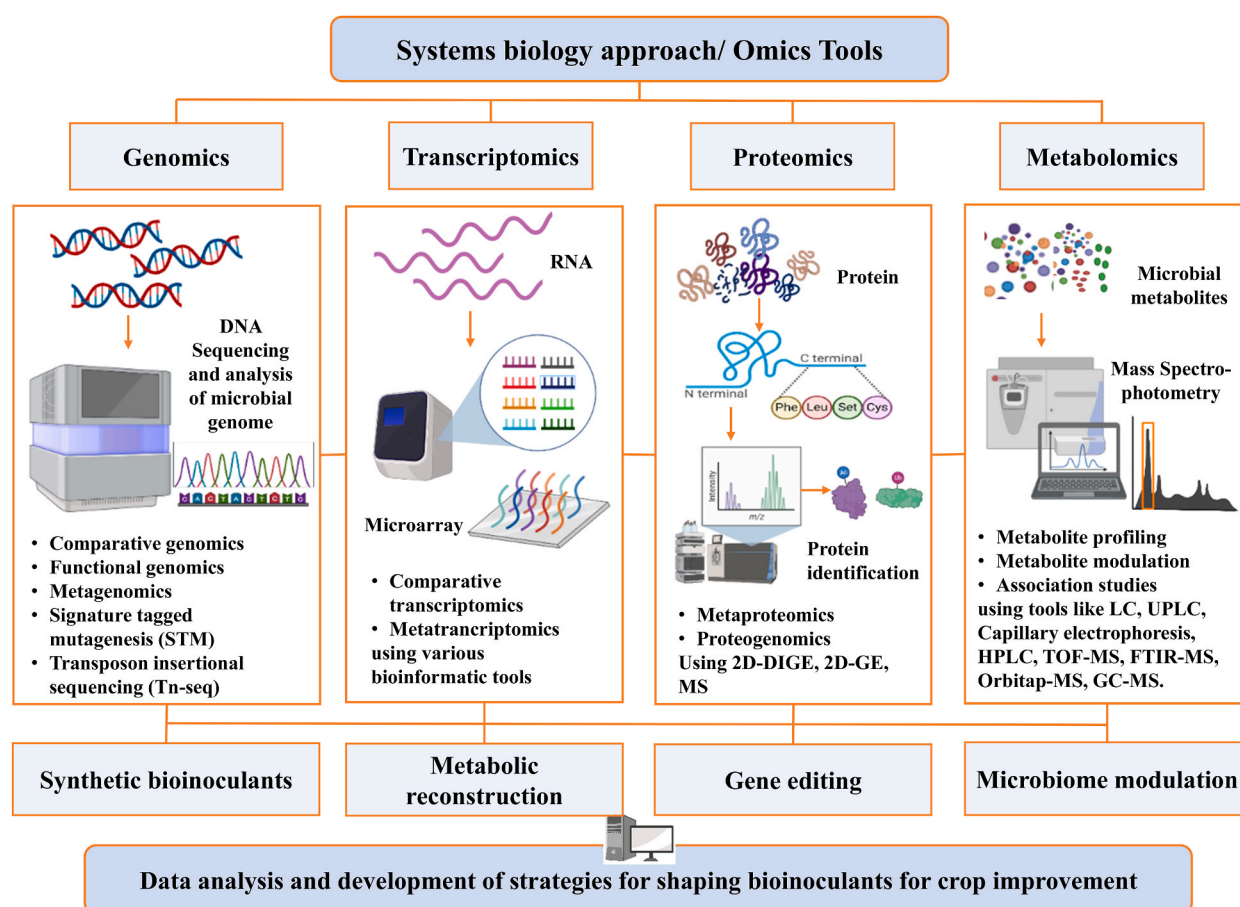


Fig. 5. Systems biology approach/Omics tools in identification and shaping of microbial bioinoculants (Created with BioRender.com).

active enzymes (CAZymes), small secreted proteins (SSPs), and indole clusters, which aid in adaptation to symbiotic environmental circumstances such as utilizing alternative nutritional (carbon) sources and dealing with plant immunological responses [77]. Genomics and RNA-seq are significant tools for learning the molecular mechanisms that influence factors such as symbiosis, rhizosphere colonization, and the subsequent formulation and moulding of bioinoculants. The use of signature-tagged mutagenesis (STM) is another approach applied for identifying genes and gene functions involved in symbiosis. The STM involves tagging mutants with different transposons and analyzing them. Transposon insertional sequencing (INseq or Tn-seq) is an even better approach for identifying gene function, involving the next-generation sequencing method to pinpoint the site with a large number of insertions across the genome. Genomics data combined with other approaches like gene editing can custom shape the bioinoculants, which may improve crop traits.

3.2. Transcriptomics and metabolomics approach

The transcriptomic approach uses RNA-sequencing and microarrays to reveal genes that are differentially expressed and transcriptional regulators under various conditions like abiotic, biotic stress, adaptation, and rhizosphere colonization, etc. The meta-transcriptomic technique has also been used to analyze transcripts from environmental samples in order to investigate the multitude of genes that might have synergistic or antagonistic actions under certain conditions. Metabolomics technology enables the isolation and identification of a diverse spectrum of primary and secondary metabolites, offering a global picture of changes happening at the metabolite level. Plant-endophyte interactions are complicated and include a variety of response mechanisms. The use of a combined transcriptomic and metabolomic strategy has enabled us to get in-depth knowledge of the metabolic cross-talk and analyze the relationship between metabolites and gene expression.

Zhang et al. [78] investigated the influence of mycorrhizal fungal interaction with *Anoectochilus roxburghii*, an important Chinese medicinal herb, on growth, development, and bioactive elements such as flavonoids. Combined metabolomics and transcriptomics analyses were used, and significant modulation of the flavonoid biosynthetic pathway has been observed. *Ceratobasidium* sp. AR2 was also discovered to enhance the accumulation of five flavanol-glycosides, two flavanols, and two flavanones, and qRT-PCR validated the differential expression of flavonoid biosynthesis genes such as *PAL*, *CHS*, *GT*, and *4CL*. Another study was conducted by Sebastiana et al. [79] with a similar approach on cork oak roots colonized by *Pisolithus tinctorius*, a mycorrhizal fungus. It has been revealed in the study that the compounds putatively corresponding to carbohydrates, organic acids, tannins, long-chain fatty acids, and monoacylglycerols were depleted, whereas non-proteogenic amino acids, gamma-aminobutyric acid (GABA), and other putative defense related compounds were induced. The metabolomic study revealed a reduction in root metabolites and stimulation of metabolic pathways involved in stress defense, indicating a plant strategy to avoid an uncontrolled proliferation of fungal symbionts.

3.3. Proteomic approach

Proteomic studies are important for protein-level investigations for the purpose of improving genome annotation, characterizing the activity of individual proteins and non-coding RNAs, discovering phosphorylated proteomes, and assessing cellular responsiveness to varied environmental circumstances. The proteome provides translational information related to functionally translated expression and protein modification. This may be associated with other high-throughput approaches like transcriptomics and metabolomics, providing information about functionally translated proteins, post-translational modifications of protein structure, and protein interaction networks. The diverse role of proteomics can be used to shape bioinoculants to boost agricultural yield. In previous years, the plant-fungi symbiosis is less studied at the protein level. An integrated proteome analysis of the relationship between the endophyte *Piriformospora indica* and *Brassica napus* revealed differential protein expression involved in various processes such as metabolism, symbiotic signalling, defense responses, nutrient uptake, energy production, and the biosynthesis of essential metabolites. These are responsible for cellular homeostasis, root architectural modification, and cell remodelling, which are vital during the plant's symbiotic growth phase [80]. In another study carried out on endophytic *Piriformospora indica* and *Azotobacter chroococcum* consortia on *Oryza sativa*, co-inoculation of consortia enhanced plant growth and nutrient uptake more than inoculating individually. Proteome analysis further revealed the upregulation of proteins involved in nitrogen and phosphorus metabolism, boosting their absorption by plants [81].

Comparative transcriptomic and proteomic analyses have also been carried out to understand the association and regulation of gene and protein expression in primary and secondary metabolism. Yuan et al. [82] employed transcriptomic and proteomic analysis to study the fungal endophyte *Gilmaniella* sp. AL12 inoculation on *Atractylodes lancea*. The study revealed the beneficial contribution of the endophyte, such as increased primary metabolism, biomass, and sesquiterpenoid content due to increased photosynthesis, an expanding sink, and enhanced metabolic flux in the host. The association leads to a decrease in plant immune response that may contribute to enhanced plant-endophyte interaction. These studies demonstrate how omics technology can be used to better understand metabolic pathways and gene-protein networks, and how this combination with other analytical methods, such as mathematical modelling and other in silico approaches, can help better understand the biological processes involved in plant-microbe interactions.

3.4. Microbiome modulation

Natural microbial communities are made up of a diverse range of microorganisms with sometimes unknown roles. The creation of artificial synthetic communities that preserve the fundamental qualities of their natural counterparts is a potential technique to overcome the obstacles involved in researching natural communities [17]. The present methods for synthesizing microbial

communities are based on the functional nature of a particular microbial isolate and metabolic interactions amongst the isolates. Microbiome modulation works in many different ways, viz., stabilizing the microbial diversity, stabilizing the microbial evenness, microbiome shift, restoration of microbial disturbances/dysbiosis, shift towards beneficial taxa, and reduction of pathogen invasions [83].

Identifying a core is thus the first step in defining a ‘healthy’ community and forecasting community reactions to disruption. A core microbiome is composed of the members of two or more microbial assemblages connected with an environment, which are commonly occurring organisms that appear in all assemblages associated with a particular habitat. These are vital for the function of that type of community. So, identifying the operational taxonomic units (OTUs) is critical to unravelling the ecology of microbial consortia. Phylogenetic and functional redundancy are vital in realizing the relationship between the composition and function of microbial systems. There can be some functions that are restricted to certain taxa (sulphate reduction, nitrogen fixation), and there are some functions mainly that are diverse across microbial groups [84]. This dynamic nature is present across space and time.

The presence of core groups will be consistent, irrespective of temporal and spatial variations. It helps the recovery of community function when various agricultural management systems lead to disturbances in the core microbiota of the rhizosphere. High-throughput data from accessible plant and microbial genomes, as well as functional knowledge such as metabolic capacity and environmental suitability for stimulating plant development, may be used to develop custom microbiome-based solutions. Two approaches, community-based and function-based bioinoculant synthesis, have been reported (Fig. 6) [85]. One commercially available bioinoculant is QuickRoots®, consisting of *Trichoderma virens* and *Bacillus amyloliquefaciens*, which helps in increasing nutrient availability to maximize the yield potential [86]. Synthetic microbial communities operate like a specified system and can serve as a model system for evaluating the role of important ecological, structural, and functional elements of communities in a controlled manner.

3.5. Gene editing

Gene editing technology is a modern tool to modify/change candidate genes to get desired results. Gene editing entails the use of different genetic engineering techniques such as recombinant DNA technology, gene cloning, etc. For integrating modifications in the

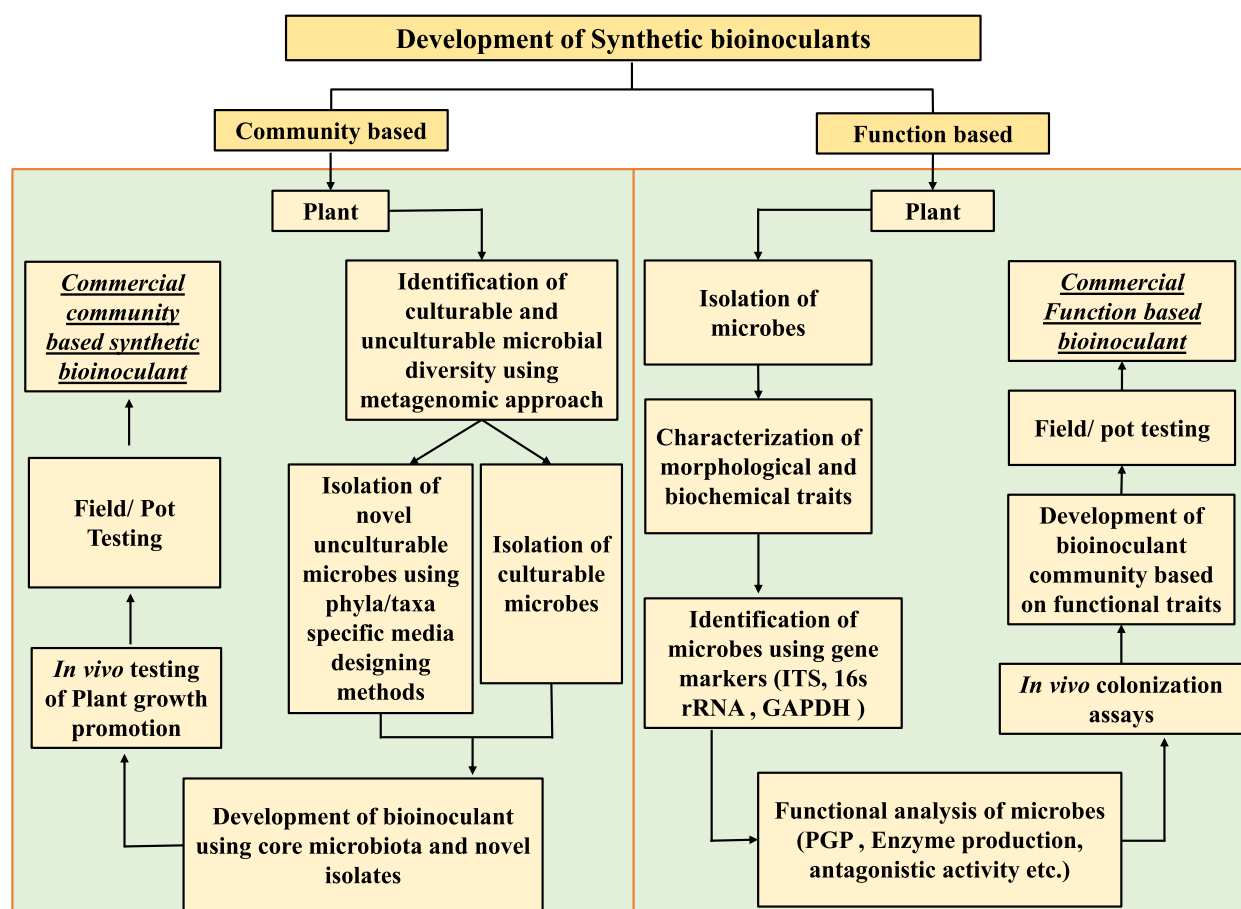


Fig. 6. Methodology for development of synthetic bioinoculants.

genomes, significant gene editing methods, including TALENs, zinc finger proteins, and clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) systems are utilised [87]. The use of gene editing technologies for fungal endophytes acting as bioinoculants can significantly increase their usefulness for crop development.

The most widely utilised CRISPR/Cas9 system of gene editing from *Streptococcus pyogenes* can be divided into three steps: recognition, cleavage, and repair. The single guide RNA or sgRNA, is composed of custom-designed CRISPR RNA (crRNA) fused with *trans*-activating crRNA (tracrRNA) and is an array of direct repeats connected by an artificial tetraloop. The CRISPR-associated protein

Table 1

Selected examples of innovative formulations used for enhancing agriculture productivity (2017–2022).

S. No.	Organism used in Bioformulation	Carrier used for formulations/Mode of inoculation	Test Plant/Disease	Beneficial Traits conferred to the host	References
1.	<i>Galactomyces geotrichum</i> WLL1	Culture broth	<i>Glycine max</i> (L.) Merr	Improved biometric and nutritional parameters	[101]
2.	<i>Rhizopus</i> sp., <i>Aspergillus fumigatus</i> , <i>Fusarium proliferatum</i> , <i>Penicillium radicum</i>	Endophytic isolates added to pot mix	<i>Lactuca sativa</i> L	Alleviates chromium toxicity	[102]
3.	<i>Fusarium redolens</i> , <i>Phialemoniopsis cornearis</i> , and <i>Macrophomina pseudophaseolina</i>	Wheat bran and Talc	<i>Coleus forskohlii</i>	Forskolin enhancement, disease management and increase in plant growth and root yield	[103]
4.	Consortium of <i>Pseudomonas aeruginosa</i> DRB1 and <i>Trichoderma harzianum</i> CBF2	Pesta granules, talc powder, alginate beads and liquid formulations	Banana wilt disease	Biocontrol efficacy against <i>Fusarium oxysporum</i> f. Sp. <i>Cubense</i> (Foc)	[104]
5.	Consortium of mycorrhizal fungi <i>Rhizoglossum irregularis</i> and <i>Funneliformis mosseae</i> , along with <i>Trichoderma koningii</i>	Liquid formulation	<i>Capsicum annuum</i> L.	Upregulation of metabolomics pathways (phytohormones, secondary metabolites and phenolic compounds) resulting in improved growth and better defense against biotic and abiotic stress	[105]
6.	<i>Trichoderma reesei</i>	Spore suspension	Banana wilt disease	Reduced severity against <i>Fusarium</i> wilt in banana and enhancement of yield and growth	[106]
7.	<i>Trichoderma afroharzianum</i> T22, <i>Azotobacter chroococcum</i> 76 A and 6-pentyl- α -pyrone – (a <i>Trichoderma</i> derived metabolite)	Carboxymethyl cellulose-based biopolymer	<i>Ocimum basilicum</i> L.	Improved growth promotion, enhanced yield and production of bioactive phenolic compounds namely, caffeic acid, rosmarinic acid and <i>p</i> -coumaric acid	[107]
8.	<i>Exserohilum rostratum</i> NMS1.5 (Fungal endophyte) and <i>Glomus etunicatum</i> UDCN52867 g ⁵ (arbuscular mycorrhizal fungi)	Bio-inoculation	<i>Helianthus tuberosus</i> L.	Plant growth enhancing ability	[108]
9.	Arbuscular mycorrhizal fungus (<i>Rhizophagus irregularis</i>)	Liquid formulations applied to river sand and soil mix (2:1 v/v)	<i>Musa acuminata</i> Cavendish cv. Brail	Suppression of wilt disease in banana seedlings by induced expression of defense-related genes and improved growth parameters	[109]
10.	<i>Trichoderma asperellum</i> (MAP1)	Culture biomass and filtrate	<i>Triticum aestivum</i> L.	Reduced water logging effects by modulating ethylene and polyamine pathways in the plant resulting in increased photosynthetic efficiency, higher chlorophyll content, stomatal conductance and membrane stability	[110]
11.	Arbuscular mycorrhizal fungi and <i>Trichoderma koningii</i> .	Microbe-based biostimulant tablet	<i>Ocimum basilicum</i> L.	Increased photosynthetic efficacy and enhanced phenolic, antioxidant and biomass content	[111]
12.	<i>Trichoderma asperellum</i>	Spent mushroom substrate, along with carrier molecules, press mud and talcum powder	Wilt caused by <i>Fusarium oxysporum</i> f. Sp. <i>Lycopersici</i> in Tomato plant F1 Hybrid King 180	Significant phenological improvements in growth, disease resistance, nutritional and antioxidant contents of the tomato plant	[112]
13.	<i>Trichoderma hamatum</i>	Liquid conidial suspension	Brassica plants (kale, cabbage, leaf rape and turnip greens)	Better phytochemical and antioxidant content especially glucosinolates with productive nutraceutical characteristics	[113]
14.	<i>Stemphylium lycopersici</i>	Soil premixed with liquid spore suspension of fungal biomass	<i>Zea mays</i> (Var. Gulibathi)	Ameliorate salt stress and improve plant growth and yield	[114]
15.	<i>Fusarium oxysporum</i>	Fungal Biomass	<i>Avena fatua</i> , a weed and <i>Triticum aestivum</i> L.	Weedicide potential and growth promotion in wheat plants by upregulating phytohormone production	[115]
16.	<i>Cladosporium tenuissimum</i>		<i>Salvia miltiorrhiza</i>	Promoting secondary metabolite (tanshinone) production by upregulating key enzymes involved in tanshinone synthesis	[116]

9 (Cas9) is a type of DNA endonuclease that makes double-stranded breaks (DSBs) at a specific site (usually 5'-NGG-3', where N can be any nucleotide base) upstream to the protospacer-adjacent motif (PAM), which is a short, conserved region. In the presence of sgRNA, Cas9 forms an effector complex with sgRNA that is capable of recognizing the target DNA sequence. Cas9 induces double-stranded cleavage in the target region, allowing modification of the gene of interest by deletion or insertion into the target site. Finally, the DSBs are repaired by host cellular machinery either by non-homologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is the predominant repair mechanism, allowing repair by joining DNA fragments through an enzymatic process, whereas HDR is a highly precise mechanism of repair which requires the use of a homologous template [88].

Gene editing is already finding applications in improving agronomic traits by enhancing plant-microbe interaction. One such example is of endophytic fungus *Phomopsis liquidambaris*, in which mitogen-activated protein kinase (MAPKK) was disrupted using the CRISPR-Cas9 system, triggering the biosynthesis of flavonoids as a stress response [89]. An efficient CRISPR-Cas system has been developed in past years in many fungi like *Pestalotiopsis fici* [90], *Aspergillus niger* [91], *A. fumigatus* [92], *Trichoderma reesei* [93], etc. Gene-editing system will allow customization and construction of bioinoculants with favourable traits such as enhanced nutrient uptake, nitrogen fixation, better stress response, etc.

3.6. Other approaches

The prediction of bioinoculant capability can be carried out using various biotechnological and in silico approaches such as metabolic reconstruction, mathematical modelling, constraint-based modelling (CBM), flux balance analysis (FBA), etc. Metabolic engineering is a new approach aiding in the analysis of metabolic pathways. The process of creating an in silico model of the whole metabolism of the cell is referred to as genome-scale metabolic network reconstruction [94].

Several methodologies are used for simulation, including modelling, which connects metabolism to genetics by identifying all anticipated processes in the cell as well as the related genes, providing a useful knowledge base of an organism's metabolic properties. CBM is a mathematical method that has been empirically verified to provide both qualitative and quantitative examinations of reconstructed networks. In addition to CBM, a flux-based analytic technique based on mass conservation is used for assessing metabolite flow via a metabolic network [87].

Metabolic reconstruction involves recognizing, classifying, and connecting metabolic network components such as genes, proteins, and metabolites that participate in metabolic activity. These methods may assist in improving and getting a dynamic and quantitative perspective on numerous biological processes. The follow-up studies can utilize the simulation systems to predict metabolic changes occurring in certain conditions such as environmental stress, climate change, and biotic stress, and the generated data may be helpful in mitigating the effects in real-time scenarios. These approaches can also provide new strategies to develop genetically modified organisms (GMOs) to withstand abiotic and biotic stresses.

4. Formulations of bio-inoculants in sustainable agriculture

The bio-formulation process in agriculture involves the selection of beneficial microbial strains and a suitable carrier. The carrier is the vehicle that houses latent live microorganisms and provides a supportive niche to the microbial population. A biofertilizer is said to be a good bioformulation when it is effective, non-polluting, readily biodegradable, with high water retention capacity and sufficient shelf life [95]. Arbuscular mycorrhizal fungi (AMF) exhibit the potential to be used as biofertilizers for it enhances plant growth by facilitating the uptake of nutrients and water and making plants resistant to soilborne diseases [96]. Infective propagules of AMF in the form of spores, extraradical mycelium, and endophytic intraradical mycelium are critical for the symbiotic establishment and beneficial outcomes [97]. Also, a good carrier is required for the survival, germination, and colonization of arbuscular mycorrhizae fungi (AMF) propagules. Barazetti et al. [98] studied the effect of AMF inoculum carrying the formulation of *Rhizophagus clarus* on the seeds of soybean (*Glycine max* L.) and corn (*Zea mays* L.) impregnated on three different carriers (peat, vermiculite, and rock phosphate). The results of the study elucidated that vermiculite and peat served as the best carrier for AMF inoculation in soybean and corn plants respectively. Another study reported that inoculation of American ginseng (*Panax quinquefolius* L.) with arbuscular mycorrhizal fungi (AMF) resulted in enhanced nutrient uptake and modified the abundance and diversity of rhizosphere microorganisms [99]. Similarly, the mycorrhizal growth response (*Glomus intraradices*) on the root architecture traits of fig cultivars (Dottaeto and Natalese) has been observed in detail by Caruso et al. [100]. The study reported positive synergism between fungal inoculation and nutrient foraging by the host plant.

Table 1 summarizes the innovative fungal bioagent formulations employed directly or coated on various carrier materials for enhanced disease-free and resilient crop production.

5. Parallel effect of bacteria and archaea endophytes as bioinoculants in sustainable agriculture

In addition to fungi, bacterial endophytes have also been assessed for their functional potential as bioinoculants.

Bacterial endophytes like *Pseudomonas*, *Bacillus*, *Burkholderia*, *Stentrophomonas*, *Pantoea*, and *Microbacterium* are the most commonly found genera reported to have plant growth promoting potential [117,118]. While maintaining a stable symbiotic association with the host plant, these bacterial endophytes produce metabolites that can influence the physiological and biochemical properties of the partner [119]. They help plants improve in growth, health, and productivity by employing both direct and indirect mechanisms. In direct mechanism, they help in nutrient mobilization and biomolecule production, including the production of hormones. In indirect mechanism, they act as biocontrol agents to serve as protection systems for the host plant in the form of

mycoparasitism, nutrient depletion, hydrolytic enzymes, toxins, and induced resistance [120]. The metabolic products produced by bacterial endophytes using these mechanisms have great potential to be used as bioinoculants in sustainable agriculture systems [121]. For example, *Bacillus* and *Brevibacillus* isolated from medicinal plants have been used as inoculants in maize plants and evaluated for their plant growth promoting potential [122]. Likewise, plant growth promoting bacteria have been explored as biofertilizers for crops like *Cicer arietinum* L., *Brassica napus* L. var. Oleracea, *Vigna radiata*, *Vigna unguiculata*, and *Cajanus cajan* [102,123,124].

It has been found that unlike bacterial endophytes, only a few archaea are plant associated. As a result, understanding the diversity and function of archaeal endophytes using a culture-dependent technique is challenging [125]. Most of the endophytic archaea reported to date have been detected within the tissues of few plants using culture-independent approaches [126, 127]. To the best of our knowledge, there are no reports regarding archaeal endophytes as bioinoculants.

6. Conclusion

The microbiome is a critical aspect of the plant ecosystem associated with vital functions like biocontrol of phytopathogens, abiotic stress tolerance, nutrient acquisition, and the production of various volatile organic compounds such as enzymes, plant hormones, and secondary metabolites essential for plant development and optimum health. Our understanding of the complexities of plant-fungi interaction has grown quickly over the last few decades. Recent advances in the development of novel biotechnological techniques such as omics technology and systems biology give an unparalleled chance to design these interactions, which can offer new alternatives to already existing pesticides and chemical fertilizers, hence taking a step towards agricultural sustainability. Along with the gene editing system, the modulation of microbial consortia for beneficial features such as phosphate solubilization, nitrogen fixation, and biocontrol has been a huge accomplishment. Custom-prepared inoculants can be employed as an excellent consortium of crop productivity. Despite the progress, the use of fungal microbiomes as bioinoculants is a study under construction. The subsequent goal should be a lab-to-land approach with the sustained translation of plant-associated microbiome, interdisciplinary research, cross-training of the next generation of scientists, and sensitizing farmers for the successful adoption of these innovative technologies.

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Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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