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Biological Control of Fusarium Head Blight by Bacterial Endophytes and Reduction of Deoxynivalenol in Wheat

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Abstract

Fusarium Head Blight (FHB) is predominately caused by *F. graminearum* whose infection not only results in yield loss, but also contaminates grains with mycotoxins, such as deoxynivalenol (DON), which pose a great threat to human and animal health. Application of chemical fungicides remains the main approach to control FHB due to the lack of effective resistant wheat cultivars. Unfortunately, long-term intensive application of fungicides led to development of fungicide-resistant *F. graminearum* strains. In addition, the application of several fungicides at sub-lethal concentrations triggers mycotoxin biosynthesis. Biocontrol of FHB by wheat associated bacterial endophytes represents an alternative and more sustainable approach as part of the integrated management of FHB and mycotoxin production with reduced environmental impact.

In this review, we explore the current wheat associated bacterial endophytes that are promising candidates as biocontrol agents against *F. graminearum* and FHB and we discuss the main mechanisms of action and major antifungal compounds produced which exhibited a high efficacy in the management of FHB and DON production.

Keywords: Bacterial endophytes; Wheat associated bacteria; Biological control; *Fusarium graminearum*; Deoxynivalenol

Introduction

Fusarium graminearum Schwabe (teleomorph *Gibberella zeae* Schw. Petch.) is the main causal agent of Fusarium head blight (FHB) in wheat and small grain cereal crops. FHB is widespread worldwide, occurring in North and South America, Europe and Asia and is one of the most economically devastating fungal diseases due to reduction in grain yield. Besides the yield losses, the problem is the potential contamination of wheat grain with mycotoxins, mainly trichothecenes such as deoxynivalenol (DON) and its acetylated derivatives 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON) and deoxynivalenol 3 β–D-glucoside (DON-3-G) [1]. The intake of DON contaminated food by animals and humans can produce immunomodulation and reproductive effects, affecting cell signaling and protein synthesis and causing weight loss in animals by refusing food intake and teratogenic disorders [2]. Due to trichothecene toxicity, the Food and Drug Administration (FDA) [3] has established a limit of 1 ppm (mg/kg) in wheat products for human consumption and the European Commission set a maximum level of 0.75 ppm in cereals for direct human consumption [4]. Therefore, reduction of DON should be considered as one of the most important evaluation parameters in control of FHB. Anthesis is the most crucial time for the development of FHB, anthers being assumed as the common pathogen entry route into the plant, thus the critical stage for the infection is relatively short [5].

Different strategies have been used to reduce the impact of FHB, including crop rotation, tillage practices, fungicides application, and planting less susceptible cultivars. Resistant varieties, cultural practices and foliar fungicides are only partially effective, none of these strategies by themselves are able to reduce the impact of FHB [6,7].

Among these strategies, the use of genetic resistant cultivars is a viable option, but at present time no successful results have been achieved [8-12]. Only modest levels of resistance have been deployed in cultivars in commercial fields; the most widely

grown cultivars are often most susceptible. At present there is no wheat cultivar identified and released with complete resistance to FHB. This could be partially explained by the quantitative nature of FHB resistance, which implies the effect of many genes with a quantitative inheritance of slow genetic gain per unit of time during the development of new cultivars.

Good levels of control can be achieved with fungicides [13,14] but their efficacy differs according to the fungal species involved [15]. Unfortunately, fungicide-resistant *F. graminearum* strains have been detected in the field after long-term intensive application of fungicides. Many of these synthetic chemical fungicides are gradually becoming ineffective due to the development of resistance mutations and new physiological pathogen races. This led to the need for even higher application dosages and for alternative pesticides with different mode of action. The availability and variety of such novel pesticides are, however, currently limited and insufficient to counteract the problem of increasing resistance development. Moreover, the application of several fungicides at sub-lethal concentrations triggers mycotoxin biosynthesis [14-19]. Furthermore, the use of certain effective fungicides has been restricted in many countries because application at late developmental stages, that is, during heading and flowering, can result in chemical residues in the harvested grain. Therefore, farmers are looking for alternative substitutes to reduce growers' dependence on chemicals to fulfill the consumers demand on pesticide-free food while maintaining environmental safety. Managing FHB with environment-friendly technologies such as biological control using antagonistic microorganisms could be of great benefit in the present context, a promising additional strategy to be used as part of an integrated management of FHB and mycotoxin production.

The current review paper provides an updated overview on the state of the art of FHB biocontrol, with a special focus on the effectiveness of using endophytes bacteria as biological control agents in the management of FHB control and reduction of DON contamination in wheat.

Bacterial endophytes: what they are and what they do in the plant

Bacterial endophytes are bacteria living inside the plant for at least one part of their life cycle, without causing adverse effect. The most commonly accepted definition for endophytic bacteria is "Bacteria that are detected from inside surface-disinfected plants or extracted from inside plants and have no visibly harmful effects on the plants" [20]. The bacterial endophytes may offer many benefits to plants by acting as growth promoters and by contributing to diseases control [20,21]. The use of endophytes as biocontrol agents has some advantages in comparison to non-endophytes, such as the ability to escape UV radiations and fluctuations of temperature and moisture encountered in the phyllosphere of plants; therefore, we can assume they are well adapted to live inside the plants and can provide reliable suppression of diseases and promote growth [5,20,22-27].

The mode of action of bacterial endophytes that contribute to the control of diseases can be either direct or indirect. Examples of direct mode of action that could be used by bacterial endophytes to control *F. graminearum* are (i) the production of secondary metabolites such as lipopeptide antibiotics, phenazine derivatives and other molecules that directly inhibit *F. graminearum*, (ii) suppression of and interference with the phytopathogens by competition for the invasion sites, (iii) biofilm formation and colonization, (iv) inhibition of spores germination and growth (v) production of enzymes such as esterases that can detoxify the virulence factors and contribute to the biocontrol of the phyto pathogen [21,28,29]. Typical example of indirect mode of action is the induction of plant systemic resistance responses (induction of systemic resistance, ISR), allowing the plant to react faster and more efficiently upon subsequent pathogen attack thus improving plant health [30]. Another form of induced resistance is the systemic acquired resistance (SAR). In both SAR and ISR, the defenses of the plants are preconditioned by previous infections or treatments that provoke resistance (or tolerance) against subsequent attacks by pathogens or parasites. The physiological and biochemical differences between the two mechanisms have been well reviewed by Vallad and Goodman [31].

Biocontrol activity of bacterial endophytes against FHB on wheat

According to some recent literature summarized in Table 1, the use of bacterial endophytes as biocontrol agent of FHB in wheat has been investigated in *in vitro* tests, in greenhouse-controlled conditions and in field trials. The most investigated endophyte bacterial agents for biocontrol of FHB in wheat belong to genera *Bacillus* [20,22-25,29,32-44,66-71] and *Pseudomonas* [21,35,45-47]. In addition to these strains, *Lactobacillus* spp. members can be interesting as potential biocontrol agents since they produce bioactive compounds (organic acids, bacteriocins, phenyllactic acid, cyclic dipeptides, fatty acids) with antimicrobial properties against *Fusarium*. In addition, *Lactobacillus* spp. have been found capable of mycotoxin detoxification [29]. Also, *Cryptococcus flavescens* was reported not only as FHB biocontrol agent but also as DON detoxifier [39] and the dynamic of population in wheat heads and anthers was studied to correlate biocontrol and inoculation.

In vitro assays and trials in greenhouse and under field conditions showed that some bacteria belonging to the genera *Bacillus* and *Pseudomonas* were able to reduce *F. graminearum* growth and FHB disease [22,24,32,40,41,48-50], using the

mechanisms reported in Table 1. In agroecosystems, the most extensive studies on bacterial biocontrol agents against fungal diseases have focused on antibiosis. For example, bacterial biocontrol agents secrete lipopeptide antibiotics, phenazine derivatives, and other antifungal metabolites to directly inhibit *F. graminearum* [51].

Bacteria from the genera *Bacillus* are microorganisms that inhabit a large number of different habitats and are well known as producers of a wide array of antagonistic compounds of different structures, having between 5 to 8% of the total genome devoted to biosynthesis of secondary metabolites, including bacteriocins, antimicrobial peptides and lipopeptides, polyketides and siderophores. The most important molecules from this group are from surfactin, iturin and fengycin families, the majority of this molecules are small, heat stable, amphiphilic proteins that act against target cells mainly by interaction with the target cells on the membrane level [52]. Some cyclic lipopeptides such as iturin, surfactin, and fengycin have been identified as the prominent compounds in *B. subtilis* strains acting against *F. graminearum*. *Bacillus megaterium* (BM1) and *Bacillus subtilis* (BS43, BSM0 and BSM2) isolated from wheat grains were found able to reduce the fungal growth and spore germination of FHB, also after heat treatment, suggesting that the antifungal molecule is heat stable. In addition, *B. megaterium* BM1 successfully controlled *Fusarium* in the field reducing its incidence up to 93%. It has been proposed that fengycins are the main responsible for this activity affecting the cell membrane of *F. graminearum,* altering its permeability and resulting in release of cell contents [7].

The TrigoCor strain of *B. amyloliquefaciens* shows potential as a biocontrol agent, despite its ability of reproducibly suppressing disease in the greenhouse but failing to consistently control disease in the field. It is believed that like many *Bacillus* biocontrol strains TrigoCor produces a diverse arsenal of antifungal metabolites particularly cyclic lipopeptides of the iturin and fengycin families, to be the main mechanism through which TrigoCor and other *Bacillus* inhibit fungal spore germination and growth on plant surfaces, prior to pathogen invasion of plant tissue. It was found that there were large differences in the levels of both *Bacillus* cells and *Bacillus*-synthesized iturins on wheat spikes in the greenhouse versus the field over time, although the overall trends were similar in both settings. Despite the presence of significant *Bacillus* cell concentrations most *Bacillus* cells post-application were metabolically dormant spores resulting in a drastic decline in iturins. Greenhouse trials and antibiosis tests indicated that the lower iturin levels on wheat spikes in the field could be a major factor limiting disease control in field settings [38].

Experiments conducted in growth chambers with *Bacillus mojavensis* using seeds inoculation, seed germination and seedling emergence as indicators, showed a significant *in vitro* control of *F. graminearum* and other *Fusarium* species [23].

Bacillus subtilis RC 218 and *Brevibacillus* sp. RC 263 performed a significant and consistent biocontrol effect on FHB severity in durum wheat field trials and DON contamination in wheat flour. Reduction in FHB severity ranged from 62 to 76% and from 42 to 58% for 2010 and 2011 field trials, respectively, and DON accumulation was significantly reduced [43,53,54].

Bacillus velezensis RC218 effectively reduced FHB severity and DON accumulation on wheat flour by 39-76% [54]. Significant reduction on disease severity (43%) and deoxynivalenol accumulation (60%) were also observed in the field trials of the moderately susceptible wheat cultivar K. Liebre after *Bacillus velezensis* RC218 was applied [55].

Bacillus vallismortis ZZ185 was isolated from healthy stems of the plant Broadleaf Holly (*Ilex latifolia* Thunb) collected in Nanjing, China. Both the culture filtrate and the n-butanol extract of strain ZZ185 showed strong growth inhibition activity *in vitro* against the phytopathogens *Fusarium graminearum*. The results showed that the filtrate and extract reduced up to 50% the symptoms of wheat seedlings infected with *F. graminearum*. Antifungal compounds were isolated as a mixture of Bacillomycin D (n-C14) and Bacillomycin D (iso-C15). The strong antifungal activity implied that the endophytic *B. vallismortis* ZZ185 and its bioactive components might provide an alternative resource for the biocontrol of plant diseases [24].

Fusarium head blight of wheat and barley were successfully suppressed by *B. subtilis* JA and D1/2 strains and the antifungal activity was attributable to fengycin [56]. Similar results were reported by Mnasri, et al. [57] comparing endophytes antibiosis assay of *Pseudomonas*, *Microbacterium* and *Bacillus.* The genera *Bacillus* was the genera with the best control ability when tested for the antimicrobial activity against FHB in durum wheat.

A total of 12,854 culturable bacterial isolates were obtained from wheat heads and examined for antagonistic activity to the *F. graminearum* strain PH-1 (NRRL 31084) *in vitro*. Among them, 492 isolates across 38 genera from healthy and infected wheat head samples demonstrated various degrees of inhibitory activities against fungal growth. Sequencing data indicated that the relative abundance of bacterial genera in the microbiome was significantly altered after infection by *F. graminearum*. In particular, the population of *Pseudomonas* spp. demonstrated a nearly 10-fold increase after *F. graminearum* infection. A bacterial isolate, *Pseudomonas piscium* ZJU60, showed strong inhibitory activity against *F. graminearum* during *in vitro* co-cultivation. Moreover, the foliar spray treatment with *P. piscium* ZJU60 in a growth chamber experiment almost completely suppressed *Fusarium* with results similar to phenamacril, a fungicide widely used to control FHB in China. In field trials, ZJU60 consistently

showed a biocontrol efficacy of 50-70% against FHB and was able to significantly reduce DON production in field trials indicating to be an effective biocontrol agent for the control of FHB [21].

Paenibacillum and *Pantoea* genera were representatives of endophytic community among the isolates from wheat seeds with plant growth promotion features and biocontrol abilities, moreover *Paenibacillus* sp. displayed greater biocontrol of *F. graminearum* together with an outstanding ability to form biofilm on an inert surface [42].

Factors affecting efficiency of biocontrol in the field: challenging of biocontrol agent

Despite numerous studies reported in the literature on the use of microorganisms as potential biocontrol agents for control of FHB (Table 1), many microorganisms exhibit the same problems with consistency when they come to field applications because one of the greatest challenges for bioproducts production and application is to maintain cell viability for long period. Thus, only few have been developed as commercial products. The steps usually followed to develop bacterial endophytes-based biological control agents are summarized in Figure 1.

The relationship between disease severity and biocontrol in the field experiments demonstrated the importance of factors such as formulation efficacy and isolate aggressiveness which are very much influenced by environmental conditions. Consistent performances of biocontrol agents and efficiency of the product across crop growing seasons and the geographical area depend on several factors which must be taken into consideration for the success of the biocontrol agent such as the type of formulation (granular, powder or liquid) and method of delivery. The production system also can drastically affect the viability of biocontrol agents, especially bacteria. Several precautions need to be adopted to increase desiccation tolerance during this process to conserve viability of the biocontrol agent. Ecological and environmental factors in the site where the product will be released, geographical and agronomic origin of the bacterial endophyte strain will influence the result of the application; the most effective biological control agents are likely those that are well-adapted to the environment in which they are applied. In addition, when a microorganism is introduced into specific environments the success of its application greatly depends on the formulation which may help the biocontrol agent in term of stability, adhesiveness, shelf life and proper distribution. Other factors affecting the success of biocontrol depends on the biology of the pathogen (for example the inhibition of sclerotia germination), number of cycling during the crop season, timing of product application, storage and distribution condition.

Timing of biological control application is of great importance to effectively control the phytopathogen. Palazzini, et al., [53] applied *Bacillus subtilis* RC 218 and *Brevibacillus* sp. RC 263 at anthesis period and obtained a significant and consistent biocontrol effect on FHB severity and DON contamination in the evaluated treatments. This could be explained with the fact that anthesis is the most crucial time for the development of FHB. With respect to the fungus development, most biocontrol agents appear to be more efficient when they are applied prior to *Fusarium*. In addition, it could be convenient also applying biocontrol agents later in the season in combination with chemical controls. However, a Decision Support System (DSS) forecasting wheat disease development can help optimizing the timing of biocontrol agent application [58].

Crane, et al. [38] found that FHB disease control and DON suppression by the TrigoCor strain of *Bacillus amyloliquefaciens* is iturin dose-dependent and the dose applied in greenhouse experiments was close to the minimal iturin concentration needed for *Fusarium* inhibition in antibiosis assays. However, a significant reduction of the active molecules synthesized by *Bacillus* was observed, beyond the point of maximum efficacy, on the wheat spikes in the field. There was a lack of disease and deoxynivalenol suppression in field settings. Field tests showed that even the most concentrated iturin sample provided only slightly more than half of the inhibition of *Fusarium* obtained with a chemical fungicide registered for the control of FHB. Future research efforts should be addressed to improve disease control on wheat spikes with *Bacillus* and should focus on maintaining higher levels of iturin over critical infection periods.

There is concern about how much disease control is expected from a plant disease biocontrol product. Crop losses higher than 50% are not economically accepted. However, the limits and percentage of crop loss due to plant disease needs to take in consideration the price premium and government incentive reward given for environment friendly cultivation. The entire technology of biological control of FHB must be sustainable not only from the environmental but also from the economic point of view.

Conclusion and future perspectives

According to the Food and Agriculture Organization of the United Nations (FAO), the world population is predicted to increase beyond 8 billion by 2030, implying major challenges for agricultural sector to secure food availability a minimal raise of 50% of agricultural food production. To cope with this growing food demand, most attention is given to raise the yield per area and decrease yield losses [59]. Therefore, decreasing diseases losses and increasing the efficiency of plant disease control is expected to significantly contribute to the globally increased food demand.

The accessibility of efficient pesticides is declining due to an increasing legislation and stringent rules on pesticides use. Actually, there is a legally time restriction for spraying with

chemicals to control and manage leaf disease in wheat, with 30 days preharvest interval to be respected. Biological control of FHB with bacterial endophytes could be an alternative environmentally sustainable strategy to be added to an integrated agronomic pest management. The biological product could be sprayed onto wheat spikes either with or following a chemical fungicide, by protecting wheat against late-season infections and contributing to reduction of FHB disease, minimizing also the accumulation of the mycotoxin deoxynivalenol (DON) in the grain.

The potential of using bacterial endophytes as alternative or complementary biological control strategy is widely recognized and is considered an important tool for FHB infection management and DON reduction in wheat. However, there are concerns about the proper development and use of reliable delivery systems for endophytes to the crop, among them (i) seed coating, (ii) spraying the crop and (iii) infecting seeds through the flowers are the most considered. The efficacy of biological fungicides needs to be optimized in order to improve the reliability of biocontrol. This can be achieved mainly through the screening and identification of efficient biocontrol agents, optimal fermentation conditions, development of stable formulations and proper application conditions (Figure 1). Bacterial endophytes-based biocontrol strategies have the potential to be promising, environmentally sustainable and economically competitive.

According to information from the Web of Science database, between 2015 and 2020, more than 50 papers (excluding reviews) were published that contain the keywords "Biocontrol" followed by "Fusarium Head Blight" and "bacterial endophyte" and present evidence of the potential of bacterial endophytes to control FHB. Therefore, it is expected that in the following years the development and adoption of innovative technologies will take place.

Future research should focus on enhancing efficiency of bacterial endophytes as biocontrol agents, increasing the level and stability of bacterial key antifungal compounds on wheat surfaces in the form and amount that could be present and active particularly when the control of FHB disease is needed during long infection periods.

Also, investigation aimed at clarifying the nature of interactions of endophytic bacteria with each other, with other microorganisms in the plant and with the physiology of wheat plant is a useful and challenging topic. Moreover, the design of microbial consortia that combine different modes of action, different environmental adaptation or perhaps several different beneficial effects may assure broad-spectrum activity and reduction of the pathogenicity of *Fusarium*. Finally, the efficiency of the formulation under different environmental conditions should be enhanced.

Another challenge to the development relies on the increasing concerns about climate changes with increases in temperature and dry periods. Climate changes will decrease the available areas for cultivation. Global agricultural productivity must be implemented under environmental sustainability goals. Therefore, it is mandatory to search for more effective technologies under stressful conditions; to help mitigate the effects of climate changes with increased availability of high quality products, in addition to commitments from the governments towards more sustainable agricultural systems, the use of bacterial endophytes as biocontrol agents of pathogens is expected to significantly increase in the following years since they play a crucial role in current agriculture regarding increased crop yield and quality, and improved food safety.

Table 1: Example of reported bacterial endophyte candidates, with direct biocontrol activity, for biological control of FHB in wheat.

Endophyte species (strain)	Type of metabolite	Reference
Bacillus megaterium (Embr 9790); (BM1)	Antibiotics	[7, 22]
Bacillus pumilus	Antibiotics	[5, 60]
Bacillus subtilis	Antibiotics (iturin; iturin A, fengycin and surfactin)	[5, 7, 22, 32, 37, 41, 42, 50, 53, 54, 61, 62, 63, 64]
Bacillus vallismortis (ZZ185)	Antibiotics [Bacillomycin D (n-C14) and Bacillomycin D (iso-C15)]	[24]
Bacillus velezensis (RC 218)	Antibiotics	[54, 55]
Bacillus licheniformis	Chitinase	[69, 70, 71]
Bacillus spp.		[66, 67, 68]
Brevibacillus sp. (RC 263)	Antibiotics	[50, 64, 53]
Lactobacillus plantarum (SLG17)	Antibiotics (Plantaricin)	[29]
Lysobacter enzymogenes (C3)	Antibiotics	[65]
Pseudomonas sp AS 64.4 (U.S. Patent 7.601.346)	Phenazine-1-carboxylic acid and lytic enzymes (chitinase, β-1,3-glucanase, protease, etc.)	[49]
Pseudomonas piscium (ZJU60)	hydrogen cyanide (HCN), phenazine-1-carboxylic acid, pyoverdine and achromobactin	[21]

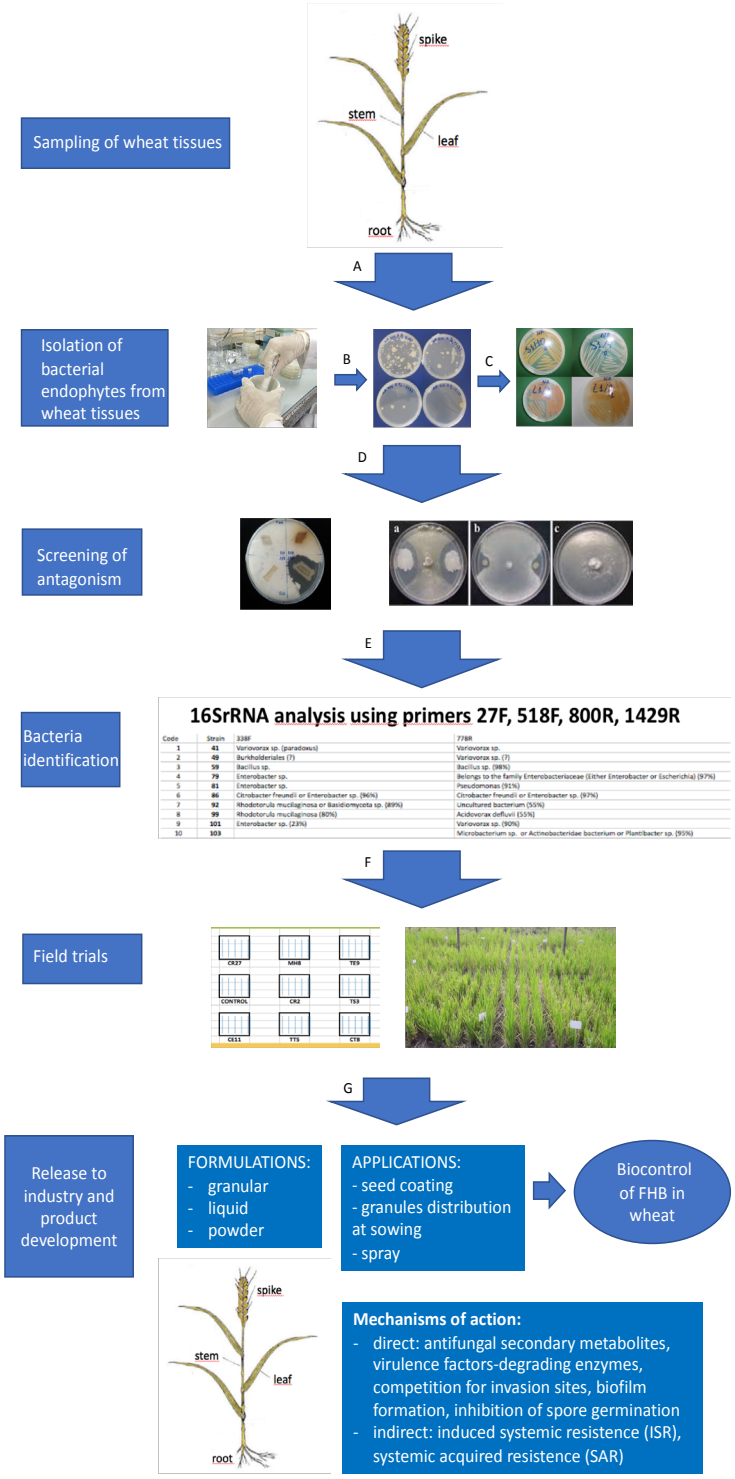

Figure 1: Process of development of bacterial endophytes-based biological control agents. Sampling of plant tissues (A), extraction of bacteria from plant tissues and growth on solid media (B), isolation of pure cultures (C). Screening of isolated bacteria for antagonistic and antifungal activity (D). Genetic identification of bacterial isolates (E). Field trials (F). Biological control product development (G).

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