6 Biofilmed Biofertilizers: Application in Agroecosystems

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Abstract

Certain soil microbiota naturally exists as surface-attached microbial communities in a biofilm mode of growth. They have been shown to be more effective at functioning than monocultures or mixed cultures of microbes. Therefore, such beneficial biofilms have been formulated *in vitro* to be used as biofertilizers called biofilmed biofertilizers (BFBFs) in agriculture and plantations. In this chapter we describe the significance of the BFBFs in addressing many issues that affect the sustainability of agroecosystems. In the literature on conventional biofertilizers, it is seen that the importance of surface attachment of microbes and biofilm formation has not been identified, though there are several other reports on the effectiveness of naturally occurring biofilms on soil particles and plant surfaces. However, the density of such biofilms on plant surfaces, particularly on the root system, is too low to have a significant effect on plant growth, as revealed by improved plant growth with BFBF applications to several crops. The BFBFs render numerous biochemical and physiological benefits to plant growth, and improve soil quality, thus leading to a reduction of chemical fertilizer (CF) NPK use by 50% in various crops. This reduction has not been achieved by conventional biofertilizers so far. The role of BFBFs is to reinstate sustainability of degraded agroecosystems through breaking dormancy of the soil microbial seed bank, and in turn restoring microbial diversity and ecosystem functioning. Thus, the concept of BFBFs is not only biofertilization, but also an holistic ecosystem approach. These formulations should therefore be considered as biofilmed microbial ameliorators (BMAs), rather than the BFBFs. If this agronomic practice were adopted in the future, it would lead to a more eco-friendly agriculture with an array of benefits to health, economics and the environment.

6.1 Introduction

Biofertilizers are live formulations of beneficial microorganisms, including nitrogen-fixing bacteria, phosphorus (P) solubilizers, algae, *Azolla* and mycorhizal fungi (Wu *et al*., 2005). They are capable of performing many tasks such as mobilizing mineral elements from unavailable forms, making atmospheric nitrogen (N_2) available to plants, suppressing pathogens and regulating

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plant growth promotion through biological processes (Tien *et al.*, 1979). Conventionally, microbiologists have paid their attention to formulate biofertilizers as monocultures or mixed cultures. The importance of using them as developed microbial communities in surface-attached biofilms was first stressed a decade ago (Seneviratne, 2003; Seneviratne and Jayasinghearachchi, 2003). In early studies, biofilms developed *in vitro* as *Penicillium* mycelium colonized by *Bradyrhizobium elkanii*, called fungal–bacterial biofilms (FBBs), were shown to result in significantly increased biological nitrogen fixation (BNF) over *B. elkanii* alone (Jayasinghearachchi and Seneviratne, 2004). Thereafter, this concept of the FBBs opened a new avenue in biofertilizer research, since subsequent studies showed that biofilms could perform improved biological functions over monocultures and mixed cultures of biofertilizers, for example in P solubilization with enhanced organic acids production (Jayasinghearachchi and Seneviratne, 2006; Seneviratne and Indrasena, 2006) and also in plant growth benefits through increased production of growth hormones (Bandara *et al.*, 2006). Later, it was reportedly realized that this was attributable to the biofilms' ability to secrete more stable extracellular substances than mixed cultures (Nadell *et al.*, 2009). These biofertilizers have now been named biofilmed biofertilizers (BFBFs) (Seneviratne *et al.*, 2008).

The importance of conventional biofertilizers as monocultures or in combination with other beneficial microbes as mixed cultures has been reviewed by Mahdi *et al.* (2010) and Saharan and Nehra (2011). Their survival and function are inconsistent under field conditions due to heterogeneity of biotic and abiotic factors and competition with indigenous organisms. Thus, they have yet to fulfil their promise and potential as commercial inoculants (Nelson, 2004). On the other hand, BFBFs have been tested successfully for their fertilizing potential of many crops, such as maize, rice, a wide range of vegetables and for plantation crops like tea and rubber, under greenhouse and field conditions. Their effectiveness under field conditions has made it possible to reduce the use of chemical fertilizer (CF) NPK by 50%, with several other beneficial functions needed for sustainability of the agroecosystems (Seneviratne *et al.*, 2011; Buddhika *et al.*, 2012a; Hettiarachchi *et al.*, 2012; Weeraratne *et al.*, 2012; Seneviratne and Kulasooriya, 2013). To our knowledge, conventional biofertilizers have not been able to achieve this CF reduction so far. The important roles of naturally existing biofilms, when attached to plant surfaces, have been discussed in relation to enhanced plant growth (Rudrappa *et al.*, 2008). Natural biofilms and their ecological significance have been reviewed widely (Davey and O'Toole, 2000; Ramey *et al.*, 2004; Rudrappa *et al.*, 2008), yet incorporation of the biofilm concept into biofertilization has not been assessed adequately, with exception of the study by Malusá *et al.* (2012) who suggest biofilms as an effective biotechnology for inoculating beneficial microbes as biofertilizers. Therefore in this chapter we describe the significance of biofertilizers in biofilm mode in addressing many issues that affect the sustainability of agroecosystems.

6.2 Biofertilizers and the Community Approach of Microbes

The major problem which is faced by current agricultural practices is the creation of undesirable ecological consequences, as many have reported (Choudhury and Kennedy, 2005; Sönmez *et al.*, 2007; Seneviratne, 2009; Savci, 2012). These have prompted research into harmless inputs for the sustainability of agroecosystems. Therefore, biofertilizers and organic farming systems in crop cultivation have got attention in safeguarding the soil and producing better quality crop products. The use of biofertilizers has several advantages over conventional chemicals used for agricultural purposes. Generally, biofertilizers are applied either to the seed or to the soil, or both, to accelerate microbial processes in the soil, thereby increasing the nutrient availability in the soil and regulating plant growth through biological processes. Research and applications of biofertilizers have been well documented. For example, *Azospirullum* inoculants were able to reduce nitrogen (N) requirement by 25% in paddy, sorghum and sunflower fields (Varma, 1993). High N levels were observed in plant tissues with biofertilizer application (Bashan *et al.*, 2004). Soil inoculation of P-solubilizing bacteria helped to reduce the requirement for phosphate fertilizer as they increased P availability in the soil (Mikanová and Nováková, 2002). Production of organic acids was reported as the major cause in

P biosolubilization from unavailable nutrient sources. In biofertilizers, microbes belonging to a wide range of genera have been reported to produce plant growth regulators such as indole acetic acid (IAA), gibberellin and cytokinins, thus supporting plant growth and development (Barea *et al*., 1976; Cassan *et al*., 2009). Production of plant growth regulators (e.g. IAA) has been reported not only to be involved in plant growth promotion but also in pathogen suppression (Yu *et al*., 2009). Further, antibiosis and mycoparasitism have been identified as major biological functions of beneficial microbes, which help suppress the growth of pathogens (Badri *et al*., 2008; Bailey *et al*., 2008; Yu *et al*., 2009). Besides, such microbes improve soil properties such as organic matter content (Wu *et al*., 2005) and soil porosity by gluing soil particles together (Czarnes *et al*., 2000), which is also important in soil aggregation and stabilization (Six *et al*., 2004).

Although there have been many reports on microbial monoculture applications as biofertilizers, the importance of biofertilizers as mixed cultures or communities has started to be emphasized lately. It was shown that combined inoculations of nitrogen-fixing and P-solubilizing bacteria were more effective than using single microorganisms for providing more balanced nutrition for crops like rice (Tiwary *et al*., 1998), maize (Pal, 1998) and some other cereals (Afzal *et al*., 2005). Further, Holguin and Bashan (1996) observed that *Azospirillum brasilense* fixed more N_2 when it was grown in a mixed culture with *Staphylococcus* sp. It has been found that large communities of soil microorganisms are effectively involved in detoxification of heavy metals, converting them into non-toxic forms (He *et al*., 2010). The importance of microbial communities rather than monocultures for plant disease suppression has also been revealed (Mazzola, 2007). Metagenomic analysis of disease suppressive soils has claimed that the antagonism is caused by the wide range and high numbers of microbiota existing in the soil (Mendes *et al*., 2011). However, in the above studies, the importance of surface attachment of microbes and biofilm formation has not been identified.

Naturally, soil microbes get attached to soil particles and plant root surfaces and develop into biofilms due to microbial communication through metabolic trading and exchanging signalling molecules (e.g. quorum sensing) (Danhorn and Fuqua, 2007; West *et al*., 2007; Nadell *et al*., 2009). In addition, plant polysaccharides stimulate biofilm formation by providing a substrate for the biofilm exopolysaccharide matrix and also by inducing matrix gene expression (Beauregard *et al*., 2013). Thereby, plants tend to select biofilm-forming microbes to colonize their plant surfaces. However, the density of such naturally formed beneficial biofilms on plant surfaces, particularly on the root system, is too low to have a significant effect on plant growth (Seneviratne *et al*., 2009), as was demonstrated in several studies by increased plant growth via enhanced root colonization, when the developed biofilms were applied (Seneviratne *et al*., 2013). The improved plant growth is attributed to increased biochemical functionality of the BFBFs (Seneviratne and Jayasinghaarachchi, 2003; Seneviratne and Jayasinghaarachchi, 2005). This was illustrated by Herath *et al*. (2013) who demonstrated that biofilms had a wider array of biochemical expressions of exudates compared with the monoculture counterparts of the biofilm. The biochemical functions include hormonal, siderophores and hydrogen cyanide (HCN) production, antifungal activities, nitrogenase activity and biosolubilization of soil inorganic sources (Bandara *et al*., 2006; Herath *et al*., 2013; Triveni *et al*., 2013), which support the fertilizing potential. Regulated metabolism in biofilms through signal exchange optimizes production of plant growth-promoting hormones such as IAA (Bandara *et al*., 2006; West *et al*., 2007; Seneviratne *et al*., 2008; Triveni *et al*., 2013; Buddhika *et al*., 2014). The optimized production of IAA increases root growth, which in turn is important in enhanced nutrient uptake (Appanna, 2007). Thus, metabolism of the BFBFs can support plant growth directly and indirectly.

6.3 Role of BFBFs in Agroecosystems

Application of BFBFs was reported to restore agroecosystems that were depleted due to agronomic practices (e.g. tea cultivation; Seneviratne *et al*., 2011). This was evident from increased soil microbial biomass carbon (MBC), organic carbon, moisture retention and hence drought tolerance, and root-associated nitrogenase activity in the study. In another study, there was a positive correlation between leaf area and net photosynthetic rate of tea (Seneviratne *et al.*, 2009). Dense colonization of biofilms on root hairs has been reported to create pseudonodules fixing atmospheric N_2 through BNF, because exopolysaccharides produced in biofilms create an oxygen-restricted environment for triggering BNF (Seneviratne *et al.*, 2008). This was evident from higher root-associated nitrogenase activity in tea with the application of the BFBFs (Seneviratne *et al.*, 2011). The increased N supply in the rhizosphere with the BFBF application was reflected by increased soil NH_4^+ availability (Buddhika *et al.*, 2012b).The BFBF application also reduced NO_3^- availability (Seneviratne *et al.*, 2011), thus increasing N use efficiency, and reducing adverse effects of N on health and the environment. Soil inoculation of BFBF also increased maize root-associated nitrogenase activity approximately fourfold, compared with application of 100% CF alone application (Buddhika *et al.*, 2012b). The nitrogenase activity was positively related to leaf chlorophyll content of BFBF-applied plants (Fig. 6.1), possibly due to ample supply of biologically fixed N for chlorophyll synthesis. The nitrogenase activity also extended even up to crop maturation, and was attributed to higher colonization of nitrogen-fixing bacteria on the root surface with the BFBFs than the 100% CF alone. In India, a similarly extended nitrogenase activity in wheat was observed with cyanobacterial BFBFs in a pot experiment (Swarnalakshmi *et al.*, 2013).

The focus of sustainability of an ecosystem is the functional diversity of soil microbes, since it is central to below-ground interactions, including food webs. Soil microbial diversity has a tremendous influence on agriculture as well as on natural ecosystems. It is a well-known fact that conventional agronomic practices, particularly CF application, deplete the diverse microbiome. However, soil application of BFBFs is known to render many beneficial effects that are vital to agricultural sustainability, including increased microbial diversity (Buddhika *et al.*, 2013). The emergence of diverse microbes with BFBF application is caused by breaking dormancy of dormant microbial forms in the soil seed bank as a response to the wide array of biochemicals secreted by the biofilms (Seneviratne and Kulasooriya, 2013).

The increased microbial diversity is considered to be one of the most important indicators of soil quality (Bastidia *et al.*, 2008; Sharma *et al.*, 2011), and is also an important determinant of soil health for increased productive capacity (Fernandes *et al.*, 1997). This is because soil microbial diversity contributes to beneficial functions such as biosolubilization, mineralization (Brookes, 1995; Pankhurst *et al.*, 1995; Yao *et al.*, 2000), rhizoremediation and natural disease suppression (Sharma *et al.*, 2011). Higher disease suppression of *Rhizoctonia solani* in potato was found in plots with the highest soil microbial diversity (Garbeva *et al.*, 2004). Similarly, there were significantly lower counts of shot hole borer in the BFBF-treated plants compared with the 100% CF-treated plants in tea cultivation (Fig. 6.2). As such, it appears that natural pest or pathogen suppression is caused by the improved soil microbial diversity, which can be achieved by BFBF application. In this way, use of biocontrol agents, some of which have been reported as unsafe (e.g. Simberloff, 2012), are not required when BFBFs are applied in agroecosystems. A natural weed-control process has also been observed recently in tea-growing soils applied with BFBFs for some time (A.P.D.A. Jayasekara, 2014, unpublished data). Cyanobacterial diversity

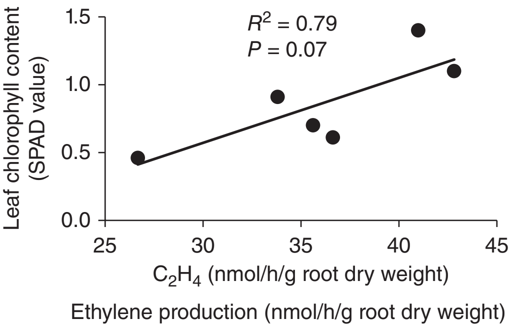

Fig. 6.1. Relationship between root-associated nitrogenase activity as evaluated by acetylene reduction of ethylene, and leaf chlorophyll content of maize plants applied with biofilmed biofertilizers (BFBFs) in a field experiment in Sri Lanka.

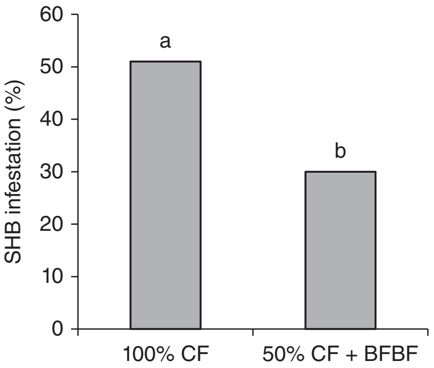

Fig. 6.2. Percentage shot hole borer (SHB) infestation of young tea plants treated with recommended chemical fertilizer (100% CF) and 50% CF + biofilmed biofertilizer (BFBF) on a tea estate at the Tea Research Institute at Talawakelle, Sri Lanka. Bars marked with different lower case letters indicate a significant difference at 5% probability level.

loss in agricultural soils has been reversed by applying cyanobacteria as monocultures (Prasanna *et al*., 2009) and/or as biofilms (Prasanna *et al*., 2011; Swarnalakshmi *et al*., 2013). These workers suggested that the utilization of a broader spectrum of biological functions of the developed cyanobacterial biofilms is the key to develop effective inocula in sustainable agriculture. However, sole application of developed FBBs as BFBFs, even in the absence of applied cyanobacterial monocultures or biofilms, has demonstrated an increased cyanobacterial diversity in a cropland soil (Buddhika *et al*., 2013) because of dormancy breaking of the soil microbial seed bank (Seneviratne and Kulasooriya, 2013). Thus, it is expected that BFBFs would increase demand for biofertilizers in the future, because they replenish the largely depleted microbiome in conventional agriculture, leading to sustainability of the agroecosystems.

BFBFs have been observed to play a vital role in agriculture starting from seed germination. Enhanced seed germination and improved seedling vigour with BFBFs compared with monoculture inoculation has been reported in several crop plants (Buddhika *et al*., 2012a; Herath *et al*., 2013; Trieni *et al*., 2013). Maize seeds tested with BFBFs for seed germination and growth showed improved performances due to regulated IAA production by the BFBFs, compared with their monoculture bacteria (Buddhika *et al*., 2014). This regulated IAA production was attributed to interactions of microbes in the biofilms, and such interactions have been reported to play amazing functions in biofilms (West *et al*., 2007). FBBs with a higher number of bacterial species, generally called higher order biofilms, were observed to pose an enhanced effect on plant growth (Seneviratne *et al*., 2009) due to their effective establishment in the soil–plant system (Swarnalakshmi *et al*., 2013). Further, collective behaviour of multiple bacterial species in biofilms has been observed to be involved in coordination, interactions and communication among the species for many ecologically important biological processes (Davey and O'Toole, 2000; West *et al*., 2007).

6.4 Fertilizing Potential of BFBFs

Different biofilms have been developed by using rhizosphere fungi and nitrogen-fixing bacteria from a wide range of genera, in order to be used as biofertilizers in agriculture and plantations (Jayasinghearachichi and Seneviratne, 2004; Seneviratne *et al*., 2011; Triveni *et al*., 2013). Application of BFBFs was first tested for soybean as a fungal–rhizobial biofilm, with increased N_2 fixation (by *c*.30%), shoot and root growth, nodulation and soil N accumulation over the application of the rhizobium alone (Jayasinghearachichi and Seneviratne, 2004). Subsequently, developed biofilms were started to be tested extensively as biofertilizers for non-leguminous crops in several agroclimatic regions of Sri Lanka (Seneviratne *et al*., 2009). Either soil or seed inoculation, or both at the same time, supplemented with 50% of the recommended CF (i.e. 50% CF + BFBF) was compared with the full dose (100%) of CF as the positive control. The 50% CF + BFBF was used here because it was confirmed from initial studies that 50% CF was the optimum level to be coupled with the BFBFs for maximizing yields in diverse soils (Seneviratne *et al*., 2009). Generally, application of BFBFs alone is not recommended, since they are fungal-bacterial biofertilizers which may incorporate a considerable fraction of plant-available soil nutrients to the fungal biomass, thus reducing plant growth. So far, the BFBFs have been tested for 12 different crops in agricultural research centres as well as farmers' fields at 25 locations covering 12 districts in the country

(Fig. 6.3). Results revealed that crop yields with 50% CF + BFBF were not significantly different (*P* > 0.05) from, and hence comparable to, yields with 100% CF (Table 6.1). This clearly shows the potential of BFBFs in reducing CF use by 50% with numerous health, economic and environmental benefits to agriculture and plantations. Widely varying soil and climatic conditions at the different locations tended to produce high variability in the yields of the same crop with the same treatment. It was reported recently that crops treated with BFBFs were limited by low levels of P in the soil (Buddhika and Seneviratne, 2014). BFBFs applied to rubber plants in the nursery also illustrated their potential in reducing CF use by 50% (Hettiarachchi *et al*., 2012). In India, applications of cyanobacteria and plant growth-promoting rhizobacteria (PGPR)-based BFBFs were observed to increase plant growth and yields of mung bean and soybean (Prasanna *et al*., 2014), and improve micronutrient biofortification in wheat (Rana *et al*., 2012a, b).

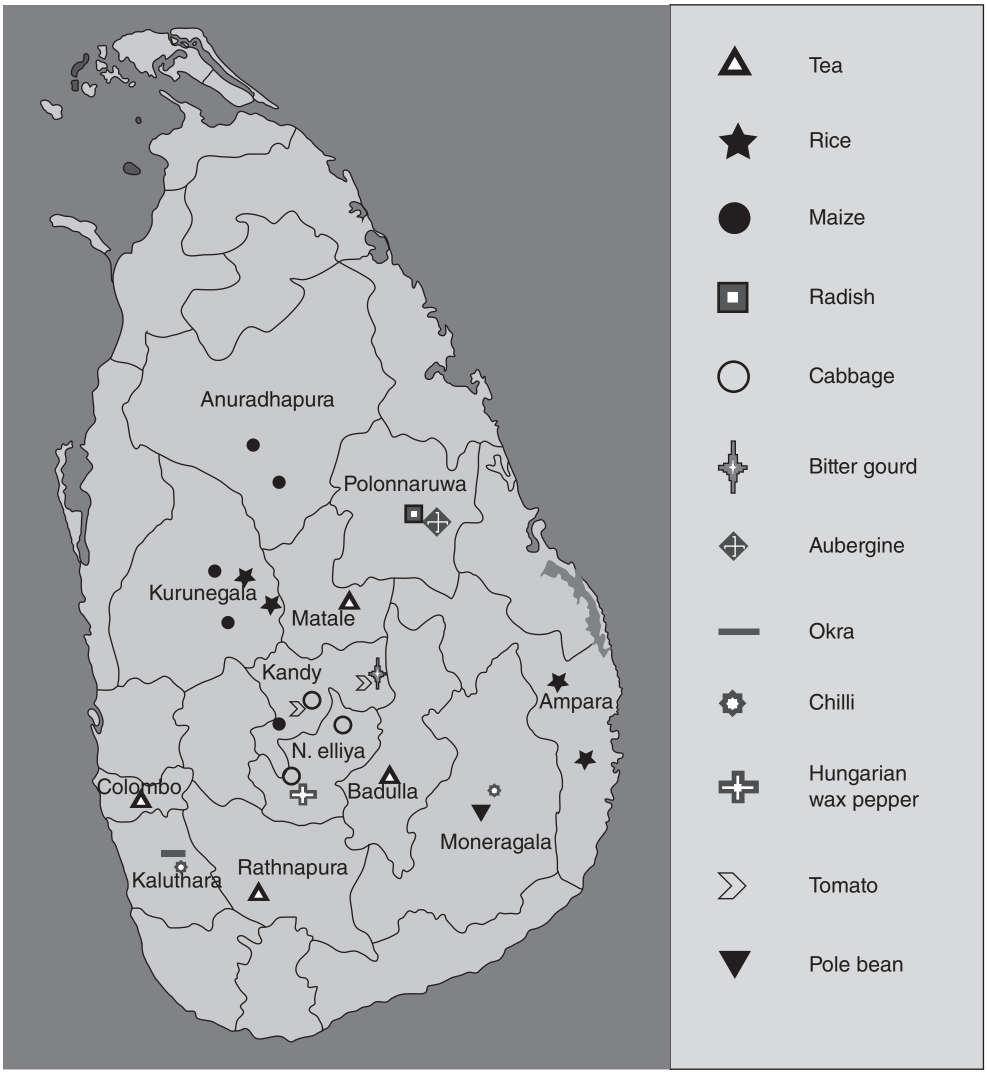

Fig. 6.3. Locations and crops of field experiments conducted with biofilmed biofertlizers (BFBF) in different districts of Sri Lanka.

Table 6.1. Mean crop yields following application of biofilmed biofertilizer (BFBF) combined with 50% of the recommended rate of chemical fertilizer (50% CF) compared with application of the recommended rate of chemical fertilizer (100% CF) in field experiments conducted in different agroecological regions of Sri Lanka.

Crop[a]	Mean ± SE crop yield (kg/ha)		Number of sites
	50% CF + BFBF	100% CF	
Tea	4300 ± 606	4100 ± 678	4
Rice	4420 ± 715	3580 ± 1295	5
Maize	2681 ± 322	2502 ± 338	3
Radish	1192 ± 251	992 ± 188	4
Cabbage	1302 ± 342	980 ± 249	4
Bitter gourd	1547 ± 445	1563 ± 440	4
Aubergine	748 ± 175	678 ± 260	4
Okra	3107 ± 1719	1739 ± 710	3
Chilli	3478 ± 1754	2350 ± 919	3
Hungarian wax pepper	238 ± 50	152 ± 39	3
Tomato	335 ± 86	397 ± 131	3
Pole bean	2762 ± 886	2396 ± 753	3

[a]Rice and maize field experiments were conducted during one or two seasons. Field experiments for vegetables were carried out during two consecutive dry and wet seasons. In the case of tea, the yields are annual averages over 4 years. In the same crop, mean yields of the two treatments were not significantly different at 5% probability level, according to Student's *t*-test.

6.5 Conclusion

The action of BFBFs differs from that of conventional biofertilizers which influence a limited set of functions such as BNF, mineral solubilization and plant growth hormone production. BFBFs show a wider range of more stable biochemical expressions and regulated metabolism for maximal effect, which are important in numerous functions of agroecosystems. BFBFs reinstate sustainability of degraded agroecosystems through breaking dormancy in the soil microbial seed bank, and in turn restoring microbial diversity and ecosystem functioning. Thus, the concept of BFBFs is an holistic ecosystem approach. BFBFs show not only enhanced biofertilization traits, but also biocontrol and other health and environment-related features. These formulations should therefore be considered as biofilmed microbial ameliorators (BMAs), rather than the BFBFs. Extensive studies conducted in various agroecosystems in the country clearly show the potential of the BMAs in reducing CF use by 50% without lowering current yields of numerous agricultural and plantation crops. If this agronomic practice was adopted in the future, it would lead to a more eco-friendly agriculture with an array of benefits to health, economics and the environment.

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