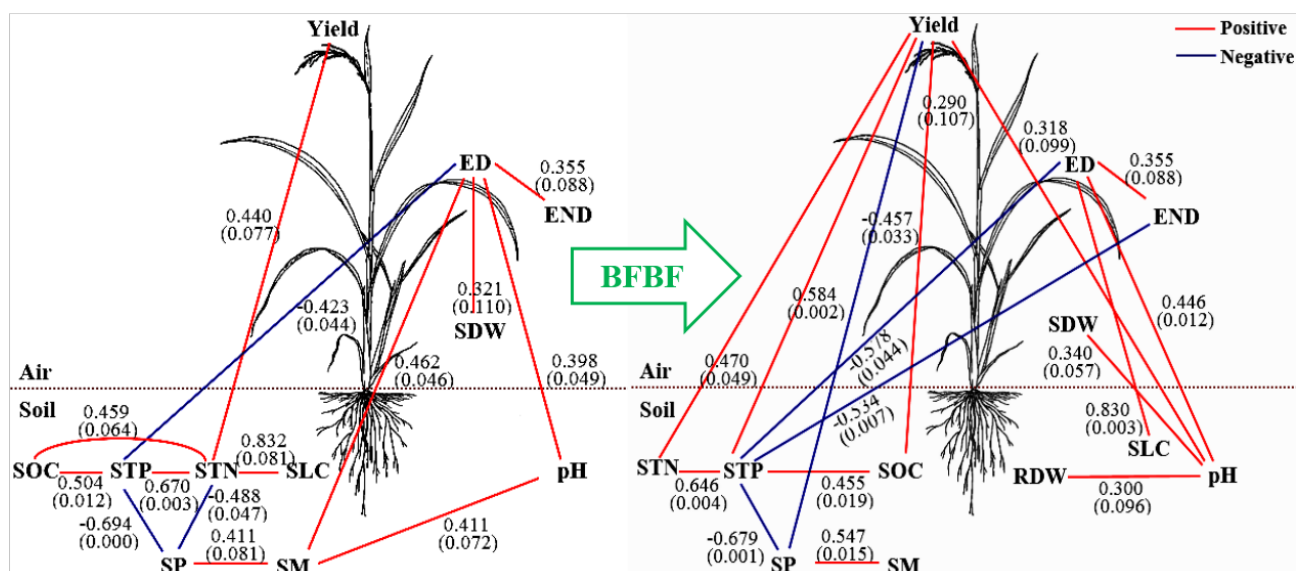


Biofilm biofertilizer can reinstate network interactions for improved rice production

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Highlights

- BFBFs contain sub network components of the agro-ecosystems.
- The sub network components help repair degraded bulk network of the agro-ecosystems.
- Thus, BFBF application helps reduce chemical fertilizers use, while increasing crop yield.
- As such, the BFBFs contribute to design eco-friendly agro-ecosystems with sustainability.

RESEARCH ARTICLE

Biofilm biofertilizer can reinstate network interactions for improved rice production

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Abstract: Biofilms are complex communities of multiple microbial species which are attached to surfaces or physical interfaces in nature. Such biofilms can also be developed *in vitro* using beneficial microbes, and can be used as Biofilm biofertilizers (BFBFs). Once applied, the BFBFs can supply sub network components to the bulk network of soil-plant-microbe parameters in agro-ecosystems degraded due to excessive use of chemical inputs. Thus, the degraded ecosystems can get their bulk network repaired through the sub network substitutions for improved interactions. Here, we analyzed selected soil, plant and microbial parameters with the application of farmers' chemical fertilizers (CF) alone practice [425 kg CF/ha (Urea 284, TSP 76 and MOP 66 kg/ha)] and BFBF practice [2.5 L of BFBF with 225 kg CF/ha (Urea 150, TSP 40 and MOP 35 kg/ha)] in 37 different locations in Sri Lanka using rice (*Oryza sativa* L.) as the test crop. Further, the data were analyzed to reveal the effect of BFBF in re-establishing networks in the agro-ecosystems. The BFBF application helped in cutting down farmers' CF use up to ca. 50%, while increasing grain yield up to ca. 25%. This was attributed to the positive effects of the BFBF towards strengthening the network interactions of the soil, plant and microbes. In this manner, BFBF practice clearly showed its potential as an eco-friendly and economically viable method to replace the farmers' current adverse practice of CF alone application. However, further studies should be conducted to collect data of a large number of variables, and they should be analyzed using more advanced methods to understand, particularly biotic and abiotic stresses for addressing them more effectively. This will eventually lead to design eco-friendly agro-ecosystems for sustainable agriculture.

Keywords: Agro-ecosystem; biofilm biofertilizers; microbial communities; networks.

INTRODUCTION

Everything in nature is interconnected or networked, directly or indirectly, and they are continuously interacting, positively (synergistically) or negatively (antagonistically). Balance of those interactions is important for stability and hence sustenance of any system (Aussenac *et al.*, 2019). In ecosystems, functional stability is strongly influenced by diverse microbial communities in the soil (Wittebolle *et al.*, 2009). There are signal-mediated interactions between

plants and microbes. Plant-microbe interactions control ecosystems, and they potentially represent a mechanistic link between plant diversity and ecosystem function (Zak *et al.*, 2003; Seneviratne, 2015).

In agro-ecosystems, modern agriculture is one of the greatest extinction threats to biodiversity (Jackson *et al.*, 2005). Tillage (Njaimwe *et al.*, 2018) and chemical inputs (Tilman *et al.*, 2002) disrupt physical, chemical and biological soil quality parameters causing network degradation and ultimately collapsing of sustainability. Chemical inputs, particularly N fertilizers and pesticides reduce microbial diversity (Van der Heijden *et al.*, 2006; Hadgu *et al.*, 2009; Pang *et al.*, 2017; Kumar *et al.*, 2018), mainly N₂ fixers. Here, microbial cells forming seeds enter in to a dormant phase in order to bypass the stress conditions like the applied chemicals (Seneviratne and Kulasooriya, 2013). Moreover, the agricultural chemicals deplete natural bio chemicals like soil enzymes (Chen *et al.*, 2018), thus relapsing protein, metabolic and signaling networks (Fox *et al.*, 2007). Thus, an urgent attention is needed to look for methods to reinstate the collapsed networks for re-establishing agro-ecosystem sustainability. Microbial interventions have been shown to be capable enough to address this issue to some extent (Alori and Babalola, 2018).

In the nature, microbes live in a variety of lifestyles such as planktonic free-living or surface-attached biofilm modes, enabling their endurance in a range of environments including extreme settings. Metabolic changes have been recognized between these two phenotypes (Favre *et al.*, 2018). It has been demonstrated that biofilm exudates contain compounds responsible for breaking dormancy of soil microbial, faunal and plant seed banks formed under stress, leading to regain lost biodiversity in degraded ecosystems (Seneviratne and Kulasooriya, 2013; Herath *et al.*, 2017). Moreover, the biofilm exudates contain protein network components (e.g. RNA, proteins etc.), and also metabolic and signaling network components (e.g. polysaccharides, QS molecules etc.), which act as sub networks of the bulk network in the soil ecosystem. As a result, when

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compared to their monoculture forms, beneficial biofilms in the rhizosphere reinstate microbe-mediated networks for enhanced cycling of nutrients and their availability to crop growth while improving crop productivity and soil fertility (Seneviratne and Jayasinghearachchi, 2005).

These biofilms occur naturally in the soil with a low density, but it is not enough to have a significant effect (Bandara *et al.*, 2006). Therefore, *in vitro* development and application of biofilms as biofertilizers, known as BFBFs is important for enriching agricultural productivity in eco-friendly manner.

Generally, BFBFs too, being developed biofilms increase microbial abundance in the soil-plant system, particularly endophytes (Seneviratne *et al.*, 2017), as explained above. Microbial endophytes play a major role in crop production, by promoting plant growth and resistivity to pathogens (Feng *et al.*, 2006). However, plants in degraded agro-ecosystems show poor endophytic diversity, leading to susceptibility to pathogens (Herath *et al.*, 2017). Thus, BFBFs act as not only biofertilizers, but also as agents to reduce biotic stress in the environment. The BFBFs contain beneficial fungal-bacterial communities in a biofilm mode (Seneviratne *et al.*, 2008a). Studies that have been carried out with BFBF application have shown that it facilitates biological N₂ fixation in non-legumes (e.g. rice), while solubilizing phosphorus and other nutrients required for crop growth via beneficial interactions between microbes and the soil (Seneviratne *et al.*, 2008a).

Rice (*Oryza sativa* L.), the most widely consumed staple food for a large part of the world's human population, is the agricultural commodity with the third-highest worldwide production. In Sri Lanka, the largest agricultural land area (34% or 0.88 million ha) is occupied by rice, being the staple food. It is cultivated as a wetland crop. About 1.8 million farm families are engaged in paddy cultivation island-wide producing 4,819 Mt rice annually (Census and Statistics, 2016). Paddy is mainly cultivated solely using chemical inputs as nutrients and agrochemicals.

Amarathunga *et al.* (2018) and Wickramasinghe *et al.* (2018) showed that the CF alone application could not support an improved plant growth and yield in rice, which could be achieved by combining CF with BFBF. A similar result was observed in the later studies (2019/2020) conducted by the Department of Agriculture, Sri Lanka. This is because the application of BFBF improves the soil-plant-microbial interactions and leads to enhanced nutrient use efficiency and increased yield while cutting down the CFs (Seneviratne *et al.*, 2008a; Premaratna *et al.*, 2018). Therefore, the present study was designed to reveal the effect of BFBF on re-establishing networks in the soil ecosystem in comparison to that of farmers' CF alone application, using rice as the test crop.

MATERIALS AND METHODS

Field sites and the experiment

The field experiments were carried out during 2018-2019 period in four major paddy growing districts of Sri Lanka;

Ampara (07° 05' N 81° 45' E, average annual temperature 27°C, elevation above sea level 37 m, average annual rainfall 1,858 mm), Kurunegala (07° 45' N 80° 15' E, average annual temperature 26°C, elevation above sea level 116 m, average annual rainfall 2000 mm), Hambanthota (06° 15' N 81° 10' E, average annual temperature 28°C, elevation above sea level 1 m, average annual rainfall 1,045 mm) and Polonnaruwa (07° 56' N 81° 0' E, average annual temperature 27 °C elevation above sea level 60 m, average annual rainfall 1,678 mm) districts consisting with variable soil types, particularly red yellow podzolic with laterite, low humic gley, non-calcic brown, reddish brown earth, solodize solonets and regosol (Ministry of Agriculture, 2014).

In all, 74 representative paddy fields (each *ca.* 0.4 ha of land area) in 37 locations spreading over thousands of hectares in Ampara (*n* = 6), Kurunegala (*n* = 28), Hambanthota (*n* = 16) and Polonnaruwa (*n* = 24) districts with diverse soil types were selected to conduct the field experiments. Two consecutive, uniformly managed paddy fields were used to apply two treatments separately. Previously, Amarathunga *et al.* (2018) and Wickramasinghe *et al.* (2018) tested a range of treatments consisting of different levels of CF alone and CF + BFBF combinations [(0, 65%, 80% and 100% of CF recommended by the Department of Agriculture (DOA), and BFBF + 65% CF and BFBF + 80% CF)]. A similar study was conducted later (2019/2020) by the DOA, Sri Lanka. They showed that the optimum level of CF that should be coupled with BFBF was 225 kg/ha. When it was coupled with BFBF, it gave a better yield than 225 kg CF/ha alone application. Thus, this was used as the recommended practice of BFBF. The treatments of the present study were (a) BFBF practice {BFBF is a fungal-bacterial biofilm (Seneviratne *et al.*, 2008b), which is now a patented [Sri Lanka patent no. 15958 (2013)] commercial product, and hence exact composition cannot be revealed due to Intellectual Property Right reasons}, [2.5 L of BFBF with 225 kg CF/ha (Urea 150, TSP 40 and MOP 35 kg/ha)], and (b) Farmers' practice [425 kg CF/ha (Urea 284, TSP 76 and MOP 66 kg/ha)]. To be realistic, farmers' CF rate was used, because in an initial survey, it was found that > 90% of the farmers do not use the CF recommendation of the DOA. Paddy was broadcasted and irrigation water was managed separately in the two fields, without mixing from surrounding fields. The BFBF was applied to the paddy fields of 0.4 ha by mixing 500 mL of BFBF with 4 L of fine sand at 2 weeks and 6 weeks after broadcasting. Our past studies showed that fine sand with CF does not show significant difference in plant growth from the CF alone application (data not shown). Therefore, in applying CF, it was not mixed with the sand. The two consecutive treatment plots were taken as a block design in each site. Thirty-seven field locations acted as replicates.

Sample collection

Two random rice hills with rhizosphere soil were uprooted carefully by digging around the root zone without damaging the root system at 50% flowering stage from each paddy field. Due to contrasting difference of plant growth between the two treatments, we limited to two plants in uprooting,

which minimized the damage to the plants growing in the paddy fields. Total sample number for each practice across the four districts was 74, which also justified the adequacy of sample number per practice. Seed samples were also collected from two random hills at physiological maturity stage.

Plant analysis

Soil was removed carefully from roots. Then, the plants were washed carefully without damaging the root system. Roots and shoots were separated and oven dried at 65 °C for constant weight, and then root dry weight (RDW) and shoot dry weight (SDW) were recorded using a top loading balance. Yield was analyzed by performing five 1 m × 1 m crop cuts in each field. Thousand grain weight (TGW) was measured using top loading balance.

Soil analysis

It is critical to ensure that the data needed to assess soil quality and health is generated by reproducible methods selected through a transparent process (Wander *et al.*, 2019). In this study, soil pH, soil moisture (SM), soil total nitrogen (STN), soil total phosphorus (STP), soil potassium (SP), soil organic carbon (SOC) and soil labile carbon (SLC) were selected as the parameters fulfilling that criterion.

In fresh soil samples, SM was determined by oven drying fresh soil at 105 °C until a constant weight. Soil pH was determined using soil:water 1:2.5 ratio. Rest of the soil samples was air-dried for analyzing other parameters mentioned above. The dried soil was grinded using mortar and pestle, and passed through 0.5 mm sieve. SOC was determined using Walkley-Black colorimetric method (Baker, 1976), whereas STN and STP were measured using distillation and titration method (Bremner and Mulvaney, 1982) and colorimetric method (Anderson and Ingram, 1993), respectively. SP was analyzed using modified Morgan extract (McIntosh, 1969) and SLC was analyzed using permanganate oxidizable carbon method (Weil *et al.*, 2003).

Microbial analysis

Endophytic diazotrophs (ED) and non diazotrophs (END) in plant leaves were enumerated by culturing them at 10⁻⁶ dilution in combined carbon medium (CCM) (Rennie, 1981) and modified CCM medium (CCM + NH₄NO₃), respectively. The surfaces of leaves were sterilized using 70% ethanol before extracting the endophytes. Colony counts were taken after 48 hours.

Data analysis

Means and correlations of all the variables of BFBF practice and farmers' CF practice were calculated. T-test was performed for mean comparison after confirmation of normal distribution of data using normality test. All data were analyzed statistically using Minitab 17 version. Network analysis was performed by Gephi software based on the correlation analysis of the parameters. Gephi has been used widely in visualizing soil-plant-microbial networks also in paddy cultivation (Bakker *et al.*, 2014; Ji

et al., 2018; Sun *et al.*, 2018). Probability level considered for statistical significance of the results was 0.10, because in agricultural field research, there is an allowance to consider the significance even up to 10% probability level (Mullen *et al.*, 2008).

RESULTS AND DISCUSSION

Soil, plant and microbial parameters

Significant increases of TGW and END were observed in the BFBF practice over the farmers' CF practice (Table 1; $p = 0.063$ and $p = 0.082$, respectively). Furthermore, RDW, SDW and yield were significantly higher in the BFBF practice than those of the farmers' CF practice (Table 1; $p = 0.017$, $p = 0.000$ and $p = 0.002$, respectively). The yield increase of the BFBF practice was ca. 24%. However, soil pH, SM, STN, STP, SP, SOC, SLC and ED were not significantly different between the two practices (Table 1; $p > 0.10$).

The yield increase of the BFBF practice over farmers' CF alone practice is possibly due to improved grain filling as reflected from increased TGW (Table 1). Enhanced plant growth with the BFBF application has contributed to this, as shown by significantly increased SDW and RDW.

Networks of BFBF and farmers' CF practices

Fifteen relationships (12 positively and 3 negatively correlated) were observed in the network of the farmers' CF practice whereas 20 relationships (13 positively and 7 negatively correlated) were observed in the BFBF practice. In the farmers' CF practice, the grain yield was directly related only to the STN ($r = 0.440$, $p = 0.077$, Figures 1 and 3). However, in the BFBF practice, five parameters *viz.* soil pH, SOC, STN, STP and SP were directly related to the grain yield ($r = 0.318$, $p = 0.099$; $r = 0.290$, $p = 0.107$; $r = 0.470$, $p = 0.049$; $r = 0.584$, $p = 0.002$ and $r = -0.457$, $p = 0.033$, respectively). Moreover, relationships between ED and STP were observed in the networks of both farmers' CF and BFBF practices ($r = -0.423$, $p = 0.044$ and $r = -0.578$, $p = 0.004$, respectively). However, separate relationships between ED and SLC, and ED and SM were noted in the BFBF and farmers' CF practices, respectively ($r = 0.830$, $p = 0.003$ and $r = 0.462$, $p = 0.046$, respectively).

In the farmers' CF practice, the grain yield was significantly limited by the STN ($p < 0.10$, Figure 1 and 3). However, in the BFBF practice, a number of soil, plant and microbial parameters controlled the yield (Figure 2 and 4). Here, plant ED association promoted the plant growth, possibly by direct and indirect mechanisms such as fixing N₂, producing plant growth hormones, improving nutrient uptake, suppressing pathogens, solubilizing phosphate and increasing plant tolerance against biotic and abiotic stresses (Mohanta *et al.*, 2010; Carvalho *et al.*, 2014). In the present study, the ED of the farmers' CF practice controlled the plant growth in the vegetative phase by influencing to SDW, which was not observed in the BFBF practice, even though there was a significantly higher SDW compared to the farmers' CF practice. This could be attributed to

important role played by the ED in supplying biologically fixed N_2 to shoot growth in the farmers' CF practice due to soil N limitation. It has also been reported that low soil N promotes diazotrophic N_2 fixation (Ai'shah *et al.*,

2009). Applied N has been efficiently utilized, and has not been limiting in the BFBF practice, as reflected from the significantly higher SDW and RDW, and also as depicted from the absence of relationships between EDs and SDW or RDW (Figure 2 and 4).

Table 1: Rhizosphere soil, plant and microbial parameters of the BFBF practice and farmers' CF practice of rice cultivations. Plant and soil samples were collected at 50% flowering whereas yield parameters were obtained at physiological maturity.

Parameter	BFBF (n = 74)	CF (n = 74)	Difference*
pH	6.35 \pm 0.13	6.32 \pm 0.09	0.03 (0.882)
SM (%)	43.61 \pm 2.84	39.79 \pm 2.00	3.82 (0.406)
STN (%)	0.23 \pm 0.01	0.18 \pm 0.01	0.05 (0.178)
STP (%)	0.36 \pm 0.06	0.41 \pm 0.07	0.05 (0.611)
SP (cmol/kg)	0.42 \pm 0.06	0.37 \pm 0.06	0.05 (0.677)
SOC (%)	1.79 \pm 0.09	1.59 \pm 0.08	0.2 (0.158)
SLC (mg/kg)	2940 \pm 501	2544 \pm 339	396 (0.520)
SDW (g/m ²)	3292.5 \pm 245	2150 \pm 135	1142.5 (0.000)
RDW (g/m ²)	1130 \pm 100	733 \pm 128	398 (0.017)
TGW (g)	20.92 \pm 0.65	17.73 \pm 0.72	3.19 (0.063)
ED (CFU/mL)	191 \pm 38	106 \pm 30	85 (0.121)
END (CFU/mL)	56 \pm 5	43 \pm 5	13 (0.082)
Grain Yield (kg/ha)	5860 \pm 243	4733 \pm 173	1127 (0.002)

Mean \pm SE in each column. SE of the means was calculated using the four district means of each parameter. *Values within parentheses are probability levels at which differences are significant. Soil moisture (SM), pH, total nitrogen (STN), total phosphorus (STP), organic carbon (SOC), labile carbon (SLC), shoot dry weight (SDW), root dry weight (RDW), thousand grain weight (TGW), yield, endophytic diazotrophs (ED), endophytic non diazotrophs (END) and soil exchangeable potassium (SP) content.

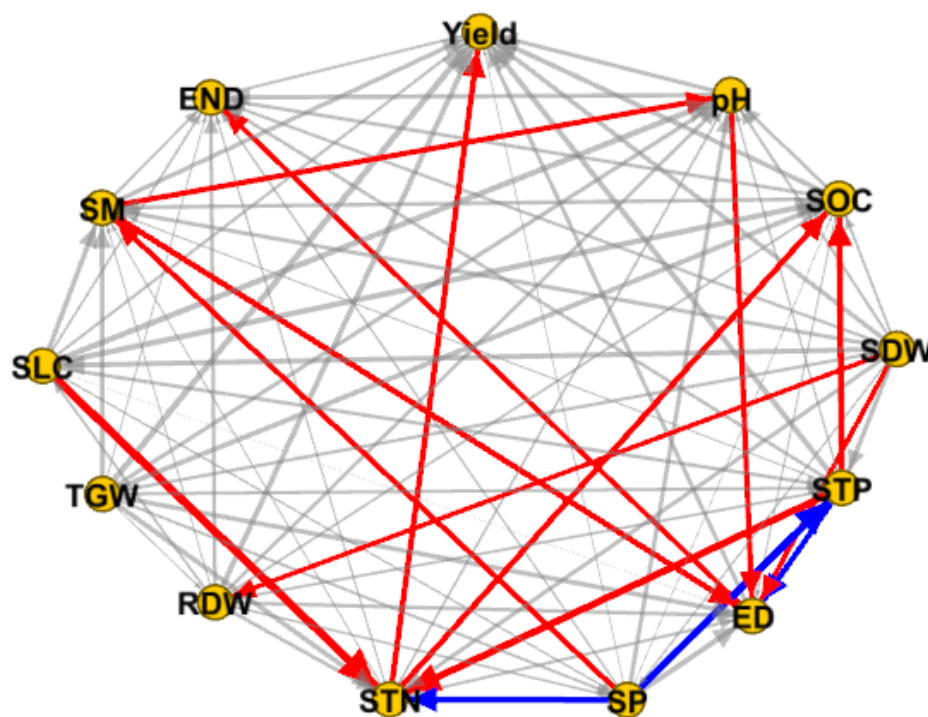


Figure 1: Network of the farmers' CF practice. Positive and negative correlations are shown in red and blue colors, respectively. The width of the arrows represents the strength of the relationships.

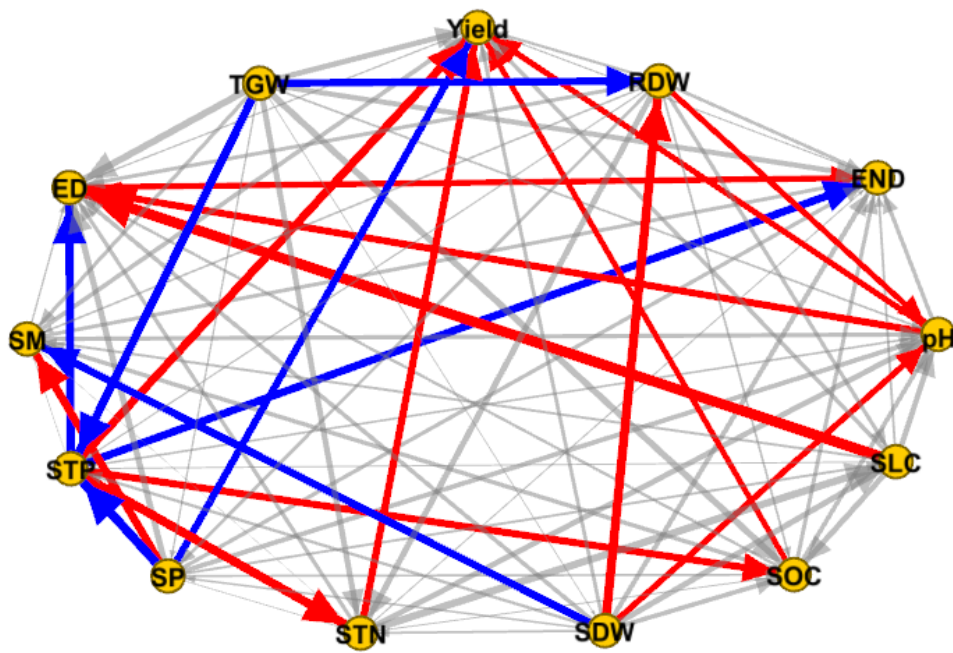


Figure 2: Network of the BFBF practice. Positive and negative correlations are shown in red and blue colors, respectively. The width of the arrows represents the strength of the relationships.

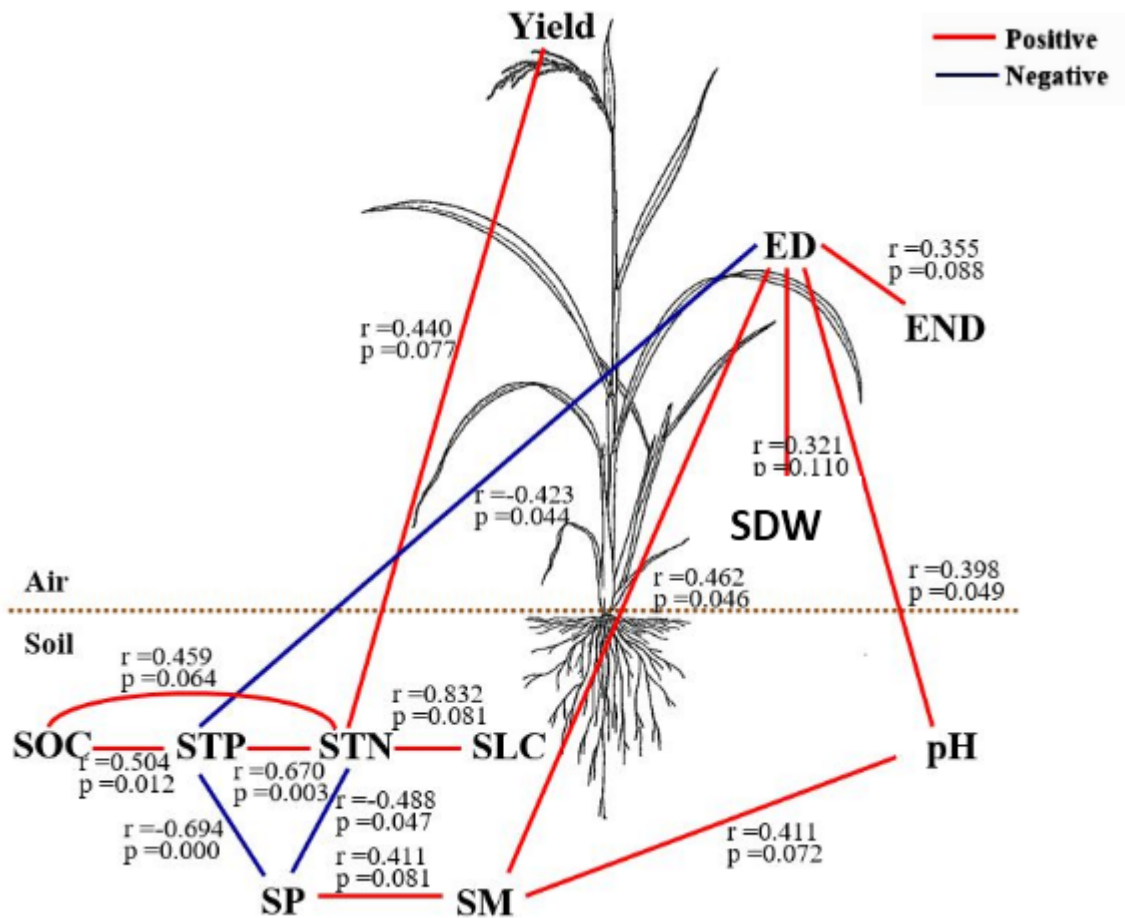


Figure 3: Opened up network of the farmers' CF practice.



Interacting sub network among STN-STP-SP-SOC-SM is common for both practices (Figure 3 and 4). In the farmers' CF practice, the grain yield has been directly controlled by the STN of the network, however in the BFBF practice, all parameters of the STN-STP-SP-SOC-SM sub network have governed the yield (Figure 3 and 4). This implies that if the STN, STP, SP, SOC and/or SM

CONCLUSION

Most of the conventional soil-plant-microbe interaction studies in agro-ecosystems are still analyzed assuming that the system variables interact univariate and/or multivariate manner. Their interaction networks are hardly established, which are of paramount importance for understanding the real action under field conditions with several, unpredictable variables. Here we show that if the STN, STP, SP, SOC and/or SM would be optimized, grain yield could be further increased only in the BFBF practice. However, to increase yield in the farmers' CF practice, STN should be further increased, which would not be favorable to soil diazotrophic action, and hence the soil-plant system. Future studies should be conducted in order to collect data of a large number of variables as much as possible, and they should be analyzed using methods like network interactions and more advanced concepts to understand, particularly biotic and abiotic stresses for addressing them

more effectively. This will eventually lead to design eco-friendly agro-ecosystems for sustainable agriculture.

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DECLARATION OF CONFLICT OF INTEREST

Authors have no conflict of interest.

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