Microbiological Properties of *Beejamrit*, an Ancient Indian Traditional Knowledge, Uncover a Dynamic Plant-Beneficial Microbial Network

Shibasis Mukherjee
Ramakrishna Mission Vivekananda Educational and Research Institute: Ramakrishna Mission Vivekananda University
Suchana Sain
Ramakrishna Mission Vivekananda Educational and Research Institute: Ramakrishna Mission Vivekananda University
Md Nasim Ali
Bidhan Chandra Krishi Viswavidyalaya: Bidhan Chandra Krishi Viswa Vidyalaya
Rupak Goswami
Ramakrishna Mission Vivekananda Educational and Research Institute: Ramakrishna Mission Vivekananda University
N Ravisankar
ICAR - Indian Institute of Farming Systems Research
Gautam Chatterjee (✉ gautamchatterjee84@gmail.com)
Ramakrishna Mission Vivekananda Educational and Research Institute: Ramakrishna Mission Vivekananda University https://orcid.org/0000-0002-9003-302X

Research Article

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Abstract

Beejamrit is an ancient organic formulation commonly used as a seed treatment in organic and natural farming in India. This low-cost input is primarily composed of cow dung, cow urine, and forest soil, which is often supplemented with limestone. In organic agriculture, it is a traditional practice among the farming community to incubate the seeds in *Beejamrit* prepared overnight before sowing in the field. However, a lack of substantial scientific evidence has been a major limitation to promote this farmers' innovative practice. On the other hand, growing data suggests that cow-based formulations are mostly enriched with microbial sources, including several plant growth-promoting rhizobacteria that are capable of synthesizing plant growth regulators. However, the microbiological properties of *Beejamrit* and their temporal changes over different periods of decomposition are largely unexplored. In this study, we aim to analyze the decomposition rate of *Beejamrit* over 7-consecutive days of incubation. This study further elucidates the microbial niche and their dynamics in *Beejamrit*, including the plant-beneficial bacteria. It was observed that the population of plant-beneficial bacteria such as the free-living nitrogen fixers and the phosphate solubilizers proliferates progressively up to 4-days of incubation. Similarly, the indolic compounds, including indole acetic acid, were enriched to the highest concentration in *Beejamrit* after 4-days of decomposition. Together, our analyses establish that *Beejamrit* provides a dynamic, microbe-based metabolic network and acts as a plant growth regulator. In conclusion, this study recommends the *Beejamrit* preparation after 4-days of incubation in the seed treatment and foliar spray to receive its optimum benefits.

Introduction

The journey of agriculture in India started long back since the Indus valley civilization and from where it eventually expanded in places in the southern part of India (Brese 1993). According to Vedic scripts and *shlokas*, several methods of crop production were narrated and believed to be practiced during this ancient period. These practices often reflect their understanding of harmonious ecological dynamics and interactions that exist between plants and soils in order to ensure long-term agricultural sustainability. In a nutshell, ancient traditional practices prevailing earlier in Indian agriculture put a greater emphasis on soil and plant health and their overall impact on agriculture, ecology, and the environment (Reviewed in Patel et al. 2020).

Since ancient times in Indian agriculture, several inputs and formulations have been documented based on traditional approaches and experiences. These formulations such as *Panchagavya*, *Sasyagavya*, *Kunapajala*, *Jeevamrit* are mostly based on local resources and are known as Indigenous Technical Knowledge (ITK). The application of these low-cost ITKs has recently been revived and re-established in modern agriculture, particularly in organic and natural farming. Furthermore, ITKs are currently regarded as the foundation for promoting agricultural sustainability in natural farming (Bharucha et al. 2020). Modern scientific understandings, on the other hand, establish that these cow-based ITKs are mostly microbe-rich concoctions. The application of these inputs to the crop field was previously believed to restore and preserve the soil ecology by fostering healthy plant-microbe interactions (Chadha et al. 2012;

Patel et al. 2020; Sharma et al. 2020). On the other hand, a few other organic formulations, such as *Beejamrit*, are thought to protect the seeds and plants from insect-disease infestation, particularly against seed-borne diseases (Chadha et al. 2012).

The term *Beejamrit* refers to *Beej* (meaning seed) dipped into *Amrit* (meaning magical liquid). It is a homemade organic input originally made up of cow dung and cow urine. The input is further enriched overnight with virgin forest soils and in some cases, with limestone (Sreenivasa et al. 2009; Sharma et al. 2021). This organic formulation is widely recommended in seed treatment to protect seeds from pathogens. In addition, *Beejamrit* has been reported to keep the young roots and rootlets away from disease-causing microbes and is so classified as an organic pesticide (Sreenivasa et al. 2009). Apart from its plausible role as a seed protectant, this organic tonic is also recommended as a foliar spray on the agricultural farm, particularly for vegetables and fruit crops (Chadha et al. 2012). It was reported earlier that the *Beejamrit* formulation is a consortium of different types of microflora, including several plant growth-promoting bacteria that are capable of producing plant growth regulators. It further suggests that the microbes in *Beejamrit* may have a direct role in antimicrobial activities as well as their role as a bio-stimulant (Sreenivasa et al. 2009). However, temporal changes in the microbial population including the plant-beneficial bacteria and the plant growth regulators such as the indole acetic acid (IAA) in *Beejamrit* after different days of its incubation are largely unknown. This scientific understanding is crucial in organic and natural farming to explore the greater benefits and better performance of the *Beejamrit* preparation. In addition, it may further ponder the inclusion of the *Beejamrit* preparation in fertilizer-based, conventional agricultural practices.

In this work, we aim to study the microbiological properties of *Beejamrit* over different days of its incubation. We also report the population dynamics of plant-beneficial bacteria such as the free-living nitrogen fixers (FNFs) and the phosphate solubilizing bacteria (PSBs). In conclusion, our study confirms that *Beejamrit* is a rich source of indole compounds including IAA, which supports its recommended application as a foliar spray and seed treatment.

Materials And Methods

Preparation of Beejamrit

The *Beejamrit* input was prepared as described before (Bishoi et al. 2017) with a minor modification by mixing the cow dung, and the cow urine, and the lime at fixed quantitates. In this study, the cow dung, and the cow urine were collected from indigenous Sahiwal cross breed cattle grazing at the Narendrapur Ramakrishna Mission Ashrama of India. In India, this is a custom to collect these ingredients from indigenous cattle breeds. As per Gurukul protocol, 5-times more limestone was added i.e., 250 gm per 20 liters of the *Beejamrit* preparation as compared to the earlier method (Bishoi et al. 2017). Then, the mixed components were further enriched with forest soils. It may be noted here that the forest soils are considered to be crucial to enriching this input with a microbial load. In this study, the forest soils were collected from Rajabhatkhawa forest village situated in North Bengal of India. The status of the organic carbon, the pH, and the microbial population of the forest soils used in this study can be found in Supplementary Table 1.

After mixing all these inputs mentioned above, the *Beejamrit* solution was incubated for different periods (0-day as control, 1-day, 2-days, 3-days, 4-days, 5-days, 6-days, and 7-days). Finally, the *Beejamrit* samples were collected after definite days of incubation and passed through a fine muslin cloth to receive the final products. The composition of *Beejamrit* can be found in Supplementary Table 2.

Estimation of the Organic Carbon

The organic carbon was determined by the standard method (Walkley and Black 1934). Briefly, samples were first oxidized in 1 (N) potassium dichromate solution and concentrated sulphuric acid. Oxidized aqueous samples were titrated against 0.5 (N) ferrous ammonium sulfate in the presence of ferroin as an indicator to score the titrated value which corresponds to oxidizable organic carbon. The conversion of titrated value to oxidizable organic carbon was calculated by using a formula mentioned in the Walkley and Black method.

Determination of Total Soluble Protein

Total soluble protein was determined by the Lowry method (Waterborg 2009). Briefly, extraction of proteins was performed in phosphate buffer (pH 7.0). The Folin ciocaltaeu reagent was then used for colorimetric analysis by the UV-Vis spectrophotometer at 660 nm wavelength.

Determination of Culturable Microbial Population

To determine the microbial population of *Beejamrit*, appropriate growth media were prepared. The details of specific growth media for a diverse group of microbes as well as their standard cultural conditions are given in Supplementary Table 3. Similarly, the plant-beneficial bacteria such as the free-living nitrogen fixer (FNFs) and the phosphate solubilizing bacteria (PSBs) were cultured and scored in terms of their colony-forming ability by following a standard method. It is noteworthy that the plates that had 50-to-100 distinct colonies were considered for calculating the microbial population.

Determination of the Available phosphorus

The available phosphorus was determined by the Olsen method (Olsen 1954). The available P was extracted by using 0.5 (M) sodium bicarbonate solution in the presence of a teaspoon of phosphorus-free activated charcoal. Next, the samples were filtered and the filtrate was subsequently neutralized by using 2.5 (N) sulphuric acid. The para-nitrophenol indicator and the Murphy-Riley reagent were added for colour development. The optical density measurement of these extractants was monitored at 530 nm by the UV-Vis spectrophotometer.

Estimation of the Indole compounds including Indole Acetic Acid (IAA)

The indole compounds of *Beejamrit* were quantitatively assayed as reported before (Salkowoski 1885). Briefly, 10 ml of the *Beejamrit* samples were taken into a falcon tube and centrifuged at 10,000 rpm for 10 minutes. Next, 2 ml of supernatant of each sample was carefully taken out and 2 drops of orthophosphoric acid were added to acidify the solution. Then, 4 ml of Salkowski's reagent (0.5 M $FeCl_3$ solution in 35% perchloric acid) was added to it in the dark and the tubes were then incubated at room temperature for 25 minutes. Finally, the colour intensity of the samples was measured by the UV-Vis spectrophotometer at 530 nm wavelength.

Statistical analysis

Samples collected after different days of incubation as mentioned before were analyzed, n = 3 in each case. The values were plotted as the mean of the three replicates with a standard error of the mean (SEM). In this study, One-way ANOVA (and nonparametric) and Dunnett's multiple comparisons tests were performed to determine statistical significance. Similarly, Pearson's correlation coefficient analysis was performed by a standard procedure using a computer-based STAR program- Statistical Tool for Agricultural Research (http://bbi.irri.org/products).

Network analysis

The visualization of the interrelationship among pH, organic carbon, total soluble protein, PSBs, FNFs, available P, and IAA was developed following the graph-theoretic approach. The bivariate correlation matrix, involving the said parameters, was used as a symmetric adjacency matrix for generating the network diagram. UCINET 6 software (Borgatti et al. 2002) was used to develop the adjacency matrix, and NetDraw software (Borgatti 2002) was used to construct the weighted graph.

Results

The Gradual depletion of organic carbon and total soluble protein in the Beejamrit solution upon days of incubation

In any biological system, organic carbon and proteins are considered to be the principal contributors to the C/N ratio. Several lines of evidence imply that the stoichiometry of the C/N ratio in the system regulates the composition of the microbial community and in particular, governs the rate of its decomposition (Ashraf et al. 2020; Zhao et al. 2018). To study the microbial dynamics, we were initially interested in analyzing the status of the organic carbon and total soluble protein i.e., nutrient sources of the *Beejamrit* solutions collected after different days of incubation. It may be noteworthy here that the *Beejamrit* preparation has been widely recommended for use after overnight incubation. In this study, we observed that the concentration of the organic carbon (in percent value) of the *Beejamrit* solution gradually gets depleted after 1-day of incubation ($P < 0.0001$) (Fig. 1a). This result strongly indicates that the organic matters decompose into simpler forms of biomolecules in the system and subsequently, gets emitted mostly as CO_2, CH_4 (Grandy and Neff 2008; Berg 2000). It is also evident from this study that the depletion of organic carbon was found to be more rapid after 2-days of decomposition ($P < 0.0001$).

Similarly, we observed that total soluble protein in the *Beejamrit* solution decreases progressively after 1-day of incubation ($P < 0.0001$) (Fig. 1b). This observation suggests the proteolytic degradation of the proteins into peptides and amino acids which are then released as NH_3, N_2O, and NO_2 from the system. Interestingly, the depletion of soluble protein was found to be more significant after 2-days of incubation as similarly observed in organic carbon decomposition ($P < 0.0001$).

Decomposition leads to pH changes in the Beejamrit solution

We next sought to determine the pH of the *Beejamrit* solutions collected after different days of incubation. It was reported earlier that pH regulates the composition of the microbial population in the system and therefore, the decomposition rate (Jin et al. 2018). This study showed a gradual fall in pH upon more days of incubation ($P < 0.0001$) (Fig. 2). The initial alkaline pH of the *Beejamrit* solution (8.51 ± 0.03) indicates the presence of cow urine and limes in the system. We further noticed that the pH of the *Beejamrit* solution becomes neutral after 7-days of incubation. Importantly, the pH of the *Beejamrit* solution approaches 7.49, which is near to physiological pH, after 4-days of incubation. It was reported earlier that the neutral pH promotes the growth of most microbes, particularly the plant-growth promoting bacteria *in vitro* (Ahemad et al. 2011). Hence, it would be fascinating to study the dynamics of the microbial population in relation to pH, organic carbon, and protein content of the *Beejamrit* input upon days of incubation.

Microbial population dynamics in Beejamrit

To identify the microbial diversity and also to determine its dynamics, the *Beejamrit* solutions collected after different days of decomposition were serially diluted in appropriate concentrations and were subsequently plated over different selective nutrient agar media by following standard methods (Please see Supplementary Table 2 for details). It was observed that the overall bacterial population of the *Beejamrit* input does not increase considerably up to 2-days of initial decomposition (Fig. 3a). However, the bacterial population multiplies significantly after 3-days of incubation ($P < 0.001$) and approaches its highest number in terms of the colony-forming unit after 5-days of incubation ($2.43 \pm 0.03 \times 10^8$). After 5-days of incubation, the bacterial population is surprisingly showing a decline. On the other hand, the populations of the actinomycetes and fungi do not change significantly up to 2-days and 1-day of incubation respectively. However, both these populations were found to be gradually decreased thereafter upon its further incubation ($P < 0.0001$) (Fig. 3b-3c). Together, these findings indicate that *Beejamrit* is a poor nutrient medium for microbial multiplication over a longer period.

Beejamrit is a rich source of plant-beneficial bacteria

Plant-beneficial microbes, such as the free-living nitrogen fixers (FNFs), the phosphate solubilizing bacteria (PSBs), the potassium solubilizing bacteria (KSBs), and the IAA producers, are known to promote plant growth (Ahemad et al. 2011; Hayat et al. 2010). In this work, we, however, focused our study on free-living nitrogen fixers (FNFs) and phosphate solubilizing bacteria (PSBs) because of their role in producing plant growth regulators such as the indole acetic acid (IAA) (Please see Discussion for details).

The study reveals that the population of free-living nitrogen fixers (FNFs) gradually grows with days of decomposition. However, its population changes substantially after 3-days of incubation ($P < 0.0001$) and it reaches a maximum after 4-days of incubation ($2.30 \pm 0.12x10^7$) (Fig. 4a). We next extend our study on phosphate solubilizing bacteria (PSBs) to elucidate their role in phosphorus mineralization into plant-available forms. Importantly, we report in this study that the level of available phosphorus content in *Beejamrit* increases gradually and reaches its peak in concentration after 4-days of incubation (151 ± 3.54 mg per ml). However, the available form of phosphorus gets depleted after 5-days of decomposition (Supplementary Fig. 1). Surprisingly, we also observed here that the population of PSBs increases significantly after 1-day of incubation ($P < 0.0001$), and it reaches a maximum after 5-days of incubation ($3.63 \pm 0.09x10^6$) (Fig. 4b). This result suggests that the population of PSBs has a strong relationship with the level of available phosphorus content in *Beejamrit*. Together, these findings indicate that the *Beejamrit* solution would yield optimum performance when applied to the crop field after 4-days of its incubation.

Beejamrit **is an excellent source of the indolic class of plant growth regulator**

It is well known that indolic compounds help to promote cell elongation and cell division and hence, regulate plant growth (Uggla et al. 1996). Tryptophan, one of the twenty natural amino acids, is a known precursor for the synthesis of indole acetic acid (IAA) *in vivo* (Ahemad et al. 2011). In this study, we report the depletion of soluble protein in the *Beejamrit* solution (Fig. 1b). We assume that the protein decomposes into simpler amino acids and hence, supplies tryptophan continuously to the system. On the other hand, it was reported that the *Beejamrit* solution is an excellent organic source for seed treatment. In addition, foliar application of *Beejamrit* has been shown to boost plant growth, particularly in vegetables and fruit development (Chadha et al. 2012; Devakumar et al. 2014). Based on these data, we next sought to determine the IAA content of the *Beejamrit* solution. The study reveals that the IAA concentration in the *Beejamrit* solution rises gradually upon days of incubation ($P < 0.0001$) (Fig. 5). It is also important to note that the IAA content of *Beejamrit* solution reaches its highest concentration (20.33 ± 0.06 µg per ml) after 4-days of decomposition (Fig. 5). This concentration of the IAA is comparable to the recommended hormonal dose of IAA sprayed in crops and also, its effect on increasing the growth and yield of mungbean as reported earlier (Karamany et al. 2019). Taken together, this study establishes that the *Beejamrit* solution is an excellent source of indolic compounds including IAA.

Discussion

In this work, we aimed to study the microbial composition of the *Beejamrit* input and its microbial dynamics over 7-consecutive days of its incubation. Microbes can be found in any ecological niche and play a significant role in carbon and nitrogen cycling (Lennon et al. 2011). In this study, we report that the bacterial population grows faster in the *Beejamrit* solution after days of incubation (Fig. 3a). However, the population indicates a decline after 5-days of decomposition. Furthermore, we also observed that the concentration of organic carbon and soluble protein i.e., nutrient sources depletes rapidly after 2-days of incubation and the rate of decomposition afterward becomes saturated gradually (Fig. 1). These results together indicate that the *Beejamrit* preparation cannot provide nutrient support for microbial multiplication over a longer period of time, at least after 5-days of incubation. On the other hand, the populations of actinomycetes and fungi started to fall steadily from the first days of incubation (Fig. 3b-3c). It may indicate either the possible antifungal activity of *Beejamrit* or the faster growth of bacteria that may impede the proliferation of actinomycetes and fungi (Sreenivasa et al. 2009). Together, these observations strongly support its application as an organic pesticide to combat seed-borne diseases, which are mostly of fungal origin.

In addition to nutrient availability and their biochemical composition, pH is another critical factor that regulates microbial metabolism, and hence, it shapes the microbial niche in the system. Interestingly, the availability of nutrients is also determined by pH (Jin et al. 2018). Hence, there may be a dynamic, cooperative network operating between the pH, and the nutrient availability, which subsequently influences microbial communities. In this study, we propose that the depletion of nutrients due to the degradation of complex sugars, and proteins cause pH changes in the *Beejamrit* solution during the initial phase of microbial decomposition. Previous studies, in support, have shown that the microbial community grown initially on complex organic molecules promotes the mineralization process, which further leads to acidification (Weintraub and Schimel 2003). During the subsequent stages of decomposition (i.e., after 3-days of incubation in this case), the nutrient-limited state and the neutral pH of the *Beejamrit* solution may promote the growth and proliferation of the free-living nitrogen fixers (FNFs) and the phosphate solubilizing bacteria (PSBs). It has been reported that the depletion of nitrogen availability in combination with various carbon forms such as sucrose, malate, mannitol promotes the growth and multiplication of the free-living nitrogen fixers (FNFs) at the rhizosphere (Reviewed in Smercina et al. 2019). Furthermore, the rapid growth of phosphate solubilizing bacteria (PSBs) degrades complex carbon molecules and generates readily energy-releasing forms of sugars. This process of degradation may compensate for the energy required for a thermodynamically unfavorable nitrogen-fixing reaction (Rojas et al. 2001). In addition, the activity of the phosphate solubilizing bacteria (PSBs) may cause a release of available phosphorus that further influences nitrogen fixation positively. It may be noted here that available phosphorus in the system is an important element that promotes nitrogen fixation (Smercina et al. 2019). On the other hand, the process of nitrogen fixation is known to enrich a variety of mild acids, which promote mineral phosphate solubilization indirectly and pH changes. This evidence collectively indicates a positive, synergistic relationship between the free-living nitrogen fixers (FNFs) and the phosphate solubilizing bacteria (PSBs). In support of this network system, our findings also imply that the *Beejamrit* solution, in terms of plant-beneficial bacterial network, improves its microbial niche in combination with the free-living nitrogen fixers (FNFs) and the phosphate solubilizing bacteria (PSBs) after 3-days of incubation (Fig. 4; Supplementary Fig. 1). In summary, we report a fascinating, dynamic network of pH, organic carbon, soluble protein content, available phosphorus content, free-living nitrogen fixers (FNFs), and the phosphate solubilizing bacteria (PSBs)in this study and how it may influence the microbial niche and the IAA content of the *Beejamrit* formulation (Please see Fig. 6 and Supplementary Table 4 for details). In the future, we anticipate that this study will further stimulate comprehensive and high-throughput microbiome investigations in combination with metabolomics analysis.

Based on traditional knowledge, it is practiced among the Indian farming community that the *Beejamrit* preparation is best to use after overnight incubation. However, there is a lack of scientific reports or evidence to support this traditional practice. We report in this work that the free-living nitrogen fixers (FNFs) and the phosphate solubilizing bacteria (PSBs) reach their maximum population ranges after 4-days and 5-days of incubation respectively. This trend in plant-beneficial bacterial dynamics is well correlated with the highest observed IAA concentration after 4-days of decomposition (Fig. 5). In fact, it is well established that several known free-living nitrogen fixers (FNFs) and phosphate solubilizing bacteria (PSBs) are capable of synthesizing plant growth regulators and also providing systematic resistance against several plant pathogens (Ahamed et al. 2008; Saharan and Nehra 2011). Taken together, our analyses indicate that *Beejamrit* is most effective in terms of plant-beneficial bacteria and the IAA content after 4-days of incubation (Fig. 5).

Conclusion

In this study, we report that *Beejamrit* is enriched with plant-beneficial bacteria, including the free-living nitrogen fixers (FNFs) and the phosphate solubilizing bacteria (PSBs). In addition, *Beejamrit* is also found to be a potent source of IAA, which is a well-known plant growth regulator. Together, this study establishes that *Beejamrit* can be used effectively as a bio-stimulant to promote seed germination, plant growth, and development. This finding further recommends the application of the *Beejamrit* preparation after 4-days of decomposition for its optimum benefits and better performance. In conclusion, our study provides scientific insight into *Beejamrit* preparation and its dynamic plant-beneficial microbial network.

Declarations

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Conflict of Interest

Authors declare no competing or conflict of interest.

Authors Contribution

Conceptualization: SM, NR & GC

Investigation and Data Generation: SM & SS

Statistical Analysis: MNA & GC

Network Analysis: RG

Funding Acquisition: NR & GC

Writing – Original Draft: GC

Writing – Reviewing & Editing: SM, SS, MNA, RG, NR & GC

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Figures

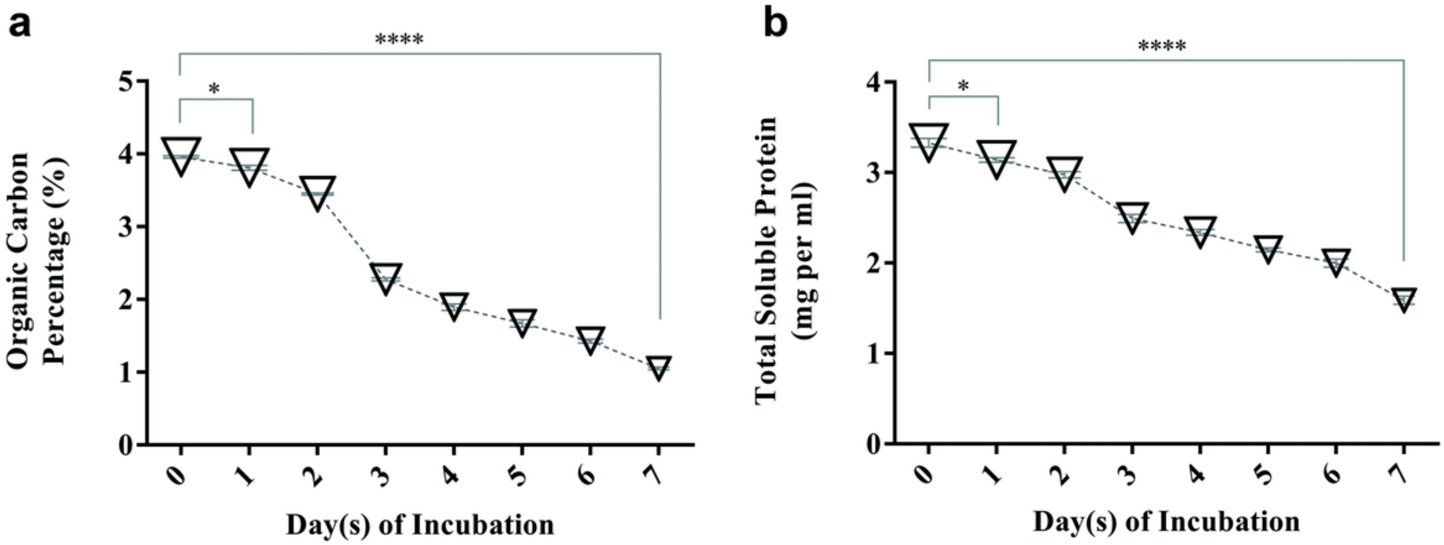

Figure 1

Estimation of biomaterials of the Beejamrit inputs indicates its gradual decomposition. The (a) organic carbon and (b) total soluble protein were estimated with three replicates with a standard error of the mean (SEM). The y-axis denotes the concentration of organic carbon in percent value, whereas the concentration in the case of total soluble protein is expressed in milligram per milliliter (mg/ml). *P<0.05; ****P<0.0001

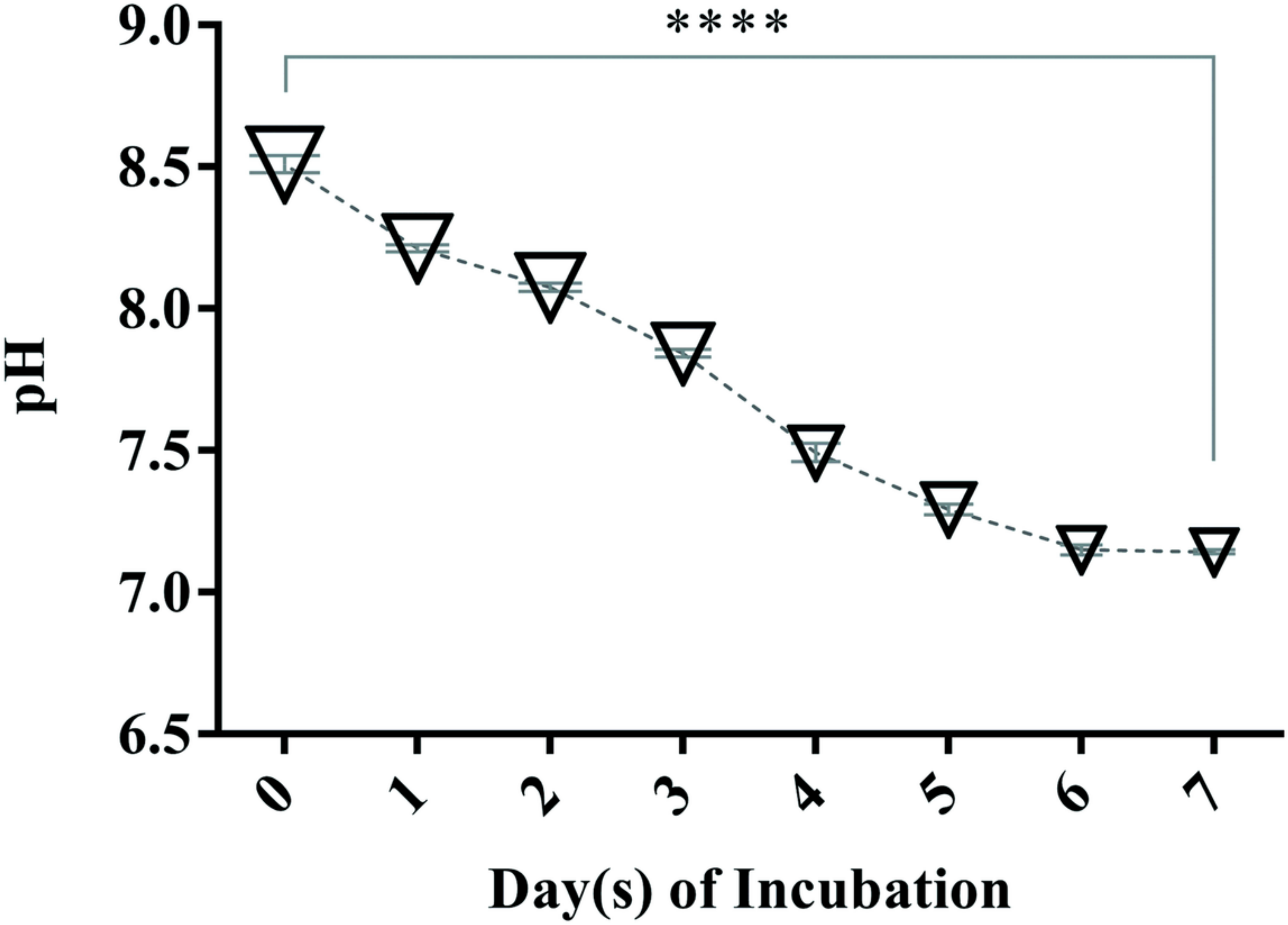

Figure 2

The pH of the Beejamrit solution decreases with days of incubation. The pH of Beejamrit of 0, 1, 2, 3, 4, 5, 6, and 7-days of incubated samples was determined with three replicates. The y-axis denotes the value of pH with a standard error of the mean (SEM). ****P<0.0001

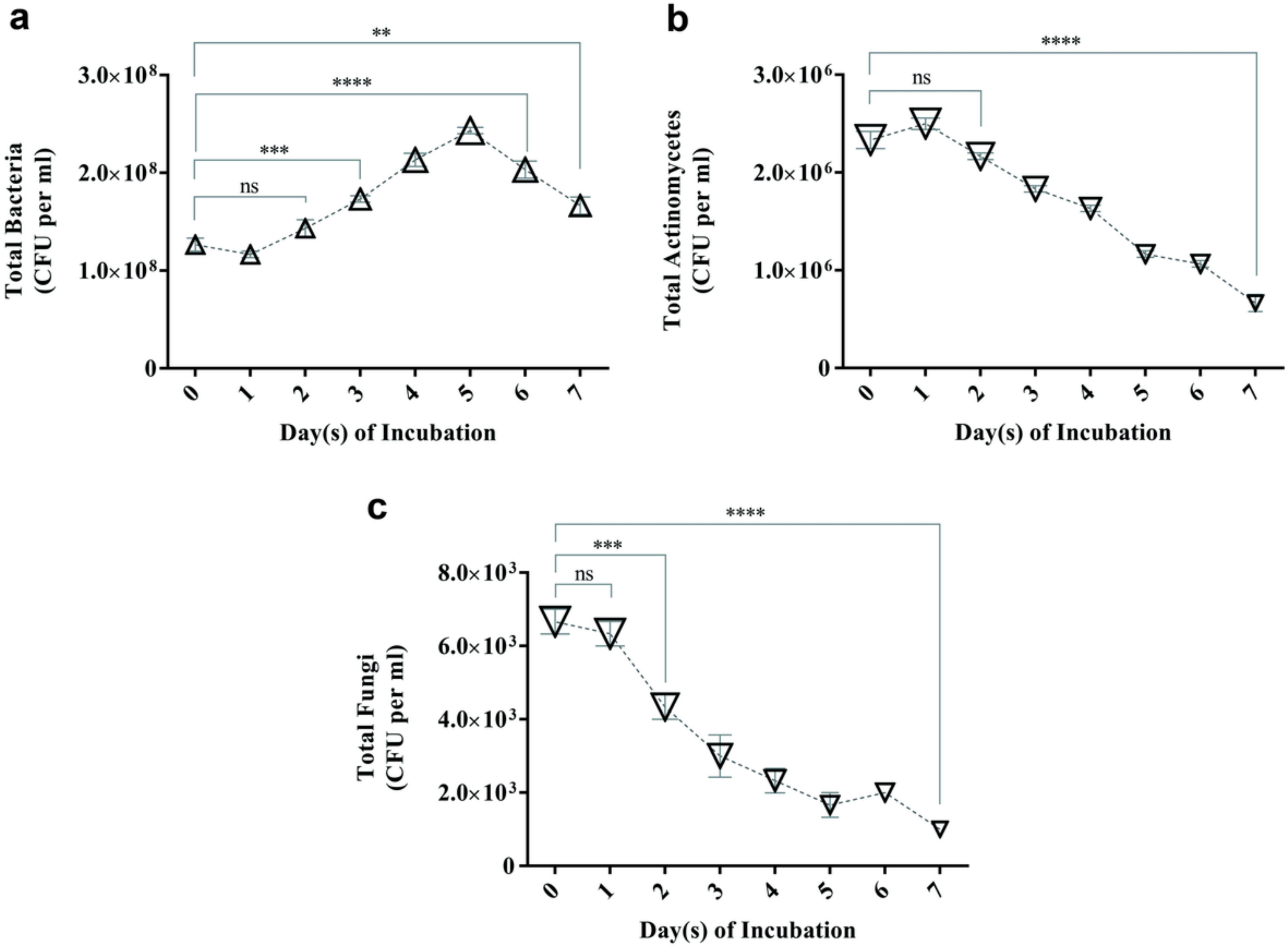

Figure 3

The diversity and dynamics of the microbial population in Beejamrit. The total culturable (a) bacteria, (b) actinomycetes, and (c) fungi count were determined with three replicates with a standard error of the mean (SEM). The y-axis denotes the population of microbes in CFU per milliliter (CFU/ml). ‘ns’ non-significant; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$

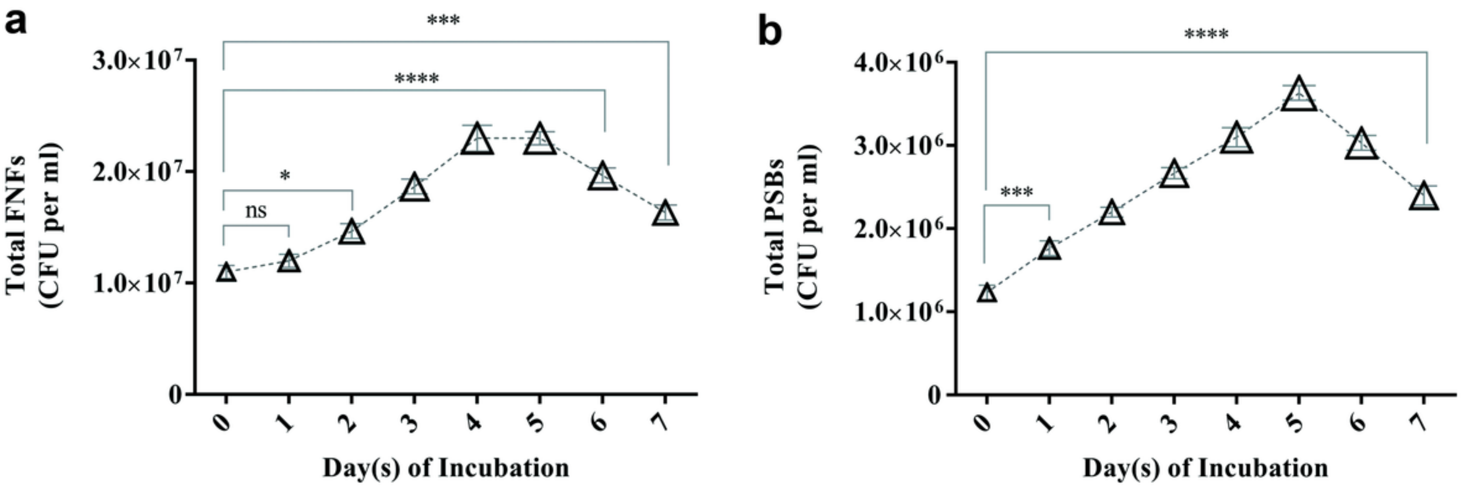

Figure 4

Dynamics of plant-beneficial bacteria in the Beejamrit input. The total culturable (a) free-living nitrogen fixers (FNFs), and (b) phosphate solubilizing bacteria (PSBs) count were determined with three replicates with a standard error of the mean (SEM). The y-axis denotes the population of microbes in CFU per milliliter (CFU/ml). 'ns' non-significant; *P<0.05; ***P<0.001; ****P<0.0001

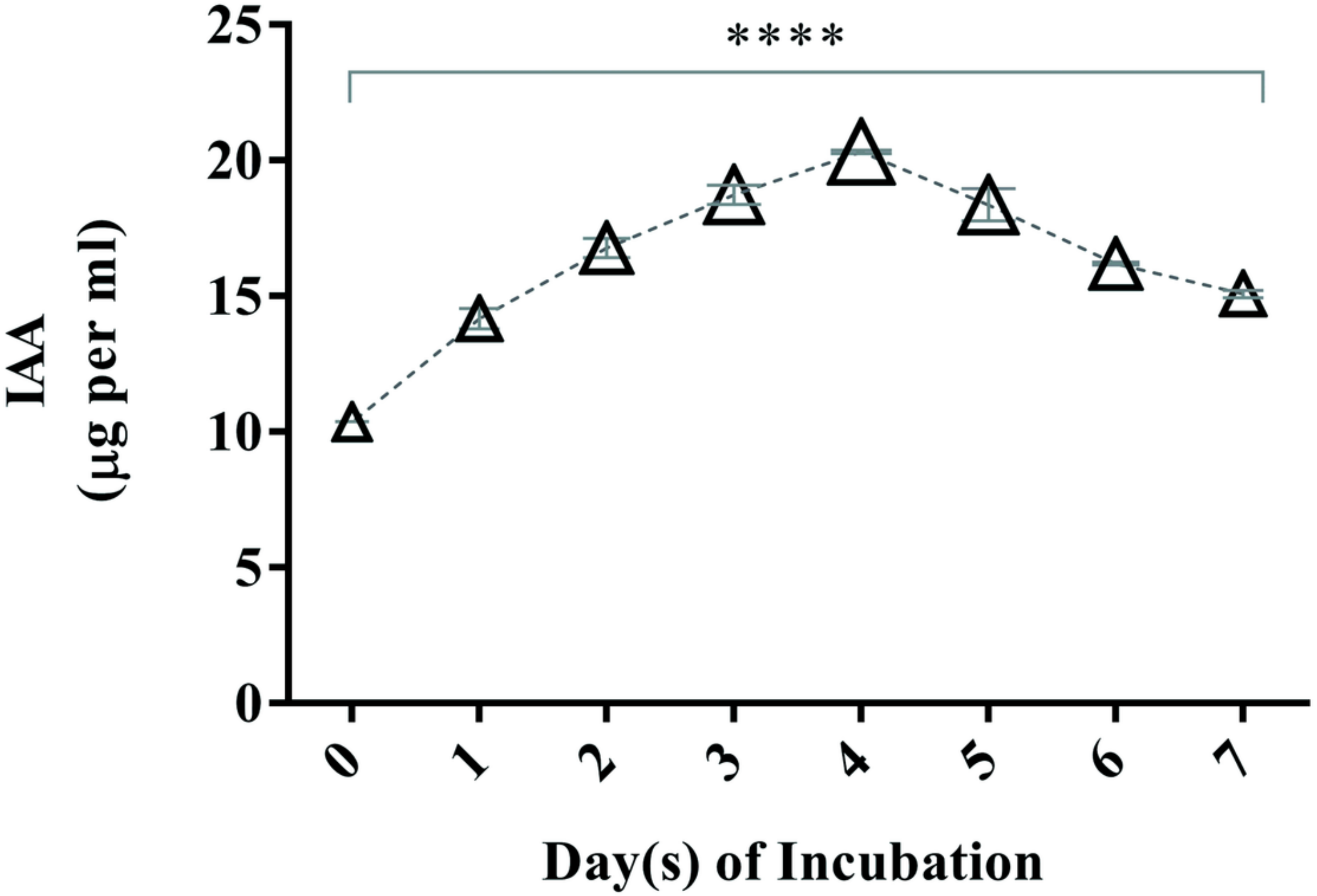

Figure 5

Determination of the IAA content of the Beejamrit solution. The IAA content of Beejamrit solution of 0, 1, 2, 3, 4, 5, 6, and 7-days of incubated samples was determined with three replicates with a standard error of the mean (SEM). The y-axis denotes the concentration of IAA in microgram per milliliter (μg/ml). ****P<0.0001

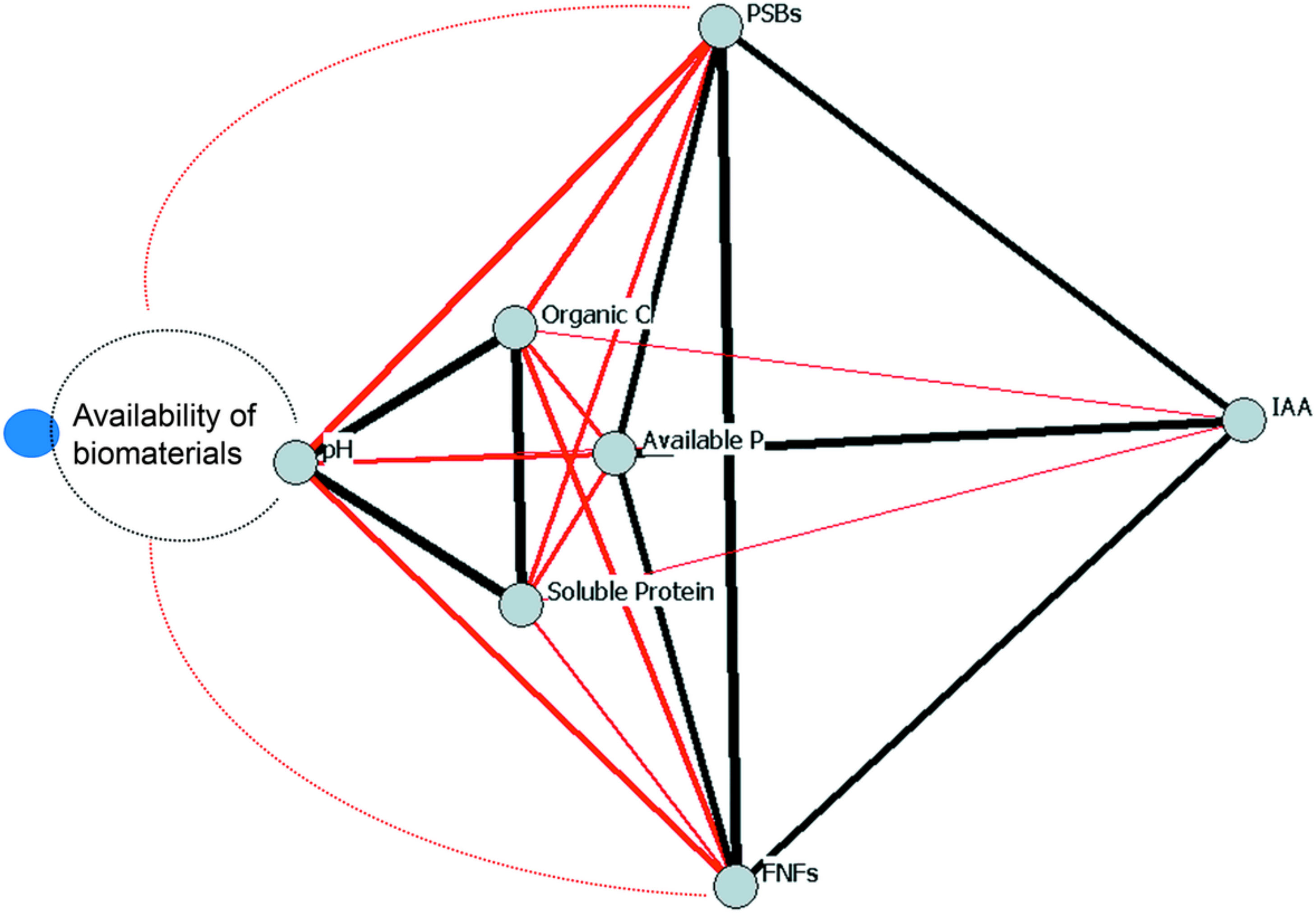

Figure 6

Interrelationship among pH, Organic C, Soluble protein, PSBs, FNFs, Available P, and IAA in the Beejamrit input. The thickness of the lines is scaled by the bivariate correlation coefficient of the concerned parameters (nodes). Black and red lines represent positive and negative relationships respectively. Here, the solid lines are based on experimental data and the dotted lines are theoretical assumptions of node relationship.

Supplementary Files

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- MukherjeeetalSuppFig.1.tif
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