



Microbiological properties of *Beejamrit*, an ancient Indian traditional knowledge, uncover a dynamic plant beneficial microbial network

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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 formulation is primarily a product of dairy excreta (e.g., cow dung and cow urine) and forest soil, often supplemented with limestone. Growing data suggest that dairy excreta are the potential sources of enriched microbial niche, including several plant growth-promoting bacteria capable of synthesizing plant growth regulators. However, the microbiological properties of *Beejamrit* and their temporal changes after different incubation periods, delineating its application in seed treatment, remain largely unexplored. Here, we aimed 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. We have shown that the population of plant beneficial bacteria, such as the free-living nitrogen fixers (FNFs) and the phosphate solubilizers (PSBs), proliferates progressively up to 4- and 5-days of incubation, respectively ($p < 0.0001$). This study also reports the total indolic content of *Beejamrit*, including indole 3-acetic acid (IAA), which further tends to oscillate in concentration based on the incubation periods incurred during the *Beejamrit* preparation. Our analyses, together, establish that *Beejamrit* provides a dynamic, microbe-based metabolic network and may, therefore, act as a plant biostimulant to crop plants. A plant-based bioassay finally demonstrates the role of *Beejamrit* in the seed treatment to improve seed germination, seedling survival rate, and shoot length trait in French beans ($p < 0.01$). In conclusion, this study highlights, for the first time, the scientific insights of *Beejamrit* as a potential seed priming agent in agriculture.

Keywords *Beejamrit* · Seed treatment · Organic farming · Plant beneficial bacteria · IAA · Microbial network

Introduction

In India, the journey of agriculture started long back since the Indus Valley civilization, and from where it eventually

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expanded to various places, including the southern part of India. According to the narration of Vedic scripts and *shlokas*, several innovations in crop production have been developed since the ancient ages and presumably provided the art and science of agricultural practices during this period. These practices often reflect their understanding of harmonious ecological dynamics and interactions between plants and soils to ensure long-term sustainability in agriculture. In a nutshell, traditional approaches in crop cultivation, prevailing during ancient India, put a greater emphasis on soil and plant health and their overall impact on agriculture, ecology, and the environment (Patel et al. 2020).

Since the ancient times of Indian agriculture, several inputs and formulations have been documented based on traditional approaches and experiences. These formulations, such as *Panchagavya*, *Sasyagavya*, *Kunapajala*, *Jeevamrit*, and several others, are primarily 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. In this current arena, ITKs have now laid the foundation for promoting agricultural sustainability in natural farming in India (Bharucha et al. 2020). Modern scientific understandings, in addition, also establish that these cow-based ITKs are mostly microbe-rich concoctions. Therefore, the application of ITKs to crop fields is likely to resurrect and maintain the soil ecology by encouraging healthy plant-microbe interactions (Chadha et al. 2012; Patel et al. 2020; Sharma et al. 2020). Besides, other known organic formulations, such as *Beejamrit*, are thought to rescue the seeds and plants from insect-disease infestation, especially against seed-borne diseases (Chadha et al. 2012).

In the Sanskrit language, *Beejamrit* refers to *Beej* (meaning seed) dipped into *Amrit* (meaning magical liquid). It is a homemade organic input primarily made up of cow dung and cow urine. This dairy excreta-based preparation is further enriched overnight with virgin forest soils, and in some cases, with limestone (Sreenivasa et al. 2009; Sharma et al. 2021). In India, the final organic formulation is widely recommended for seed treatment to protect seeds from pathogens. In addition, *Beejamrit* has also been reportedly keeping the young roots and rootlets away from disease-causing microbes and hence is classified as an organic pesticide (Sreenivasa et al. 2009). Apart from its role as a seed protectant, this organic tonic is also recommended as a foliar spray on agricultural farms, particularly for vegetables and fruit crops (Chadha et al. 2012; Devakumar et al. 2014). An earlier report has shown that the *Beejamrit* formulation is a consortium of different types of microflora, including several plant growth-promoting bacteria, and is also capable of producing plant growth regulators.

This study further suggests that the microbes in *Beejamrit* may have antimicrobial activities, indicating their role in seed treatment (Sreenivasa et al. 2009). However, temporal changes in the microbial population, including the plant beneficial bacteria and the plant growth regulators such as indole 3-acetic acid (IAA), in *Beejamrit* after different incubation periods remain largely unexplored. This scientific understanding becomes an impetus in organic and natural farming to explore the optimum benefits and better performance of the *Beejamrit* preparation. In addition, this information may also intend further to go into greater detail about the implications of *Beejamrit* in fertilizer-based, conventional agricultural practices.

In this work, we aimed to study the microbiological properties of *Beejamrit* over different days of its incubation. We also report the population dynamics of plant beneficial bacteria, including other microbes such as actinomycetes and fungi. Plant beneficial bacteria, 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 and development (Ahemad and Khan 2011; Hayat et al. 2010). This work, however, focused on free-living nitrogen fixers (FNFs) and phosphate solubilizing bacteria (PSBs) because of their additional role in IAA production. In conclusion, our study confirms that *Beejamrit* is a promising source of indole compounds, including IAA, and supports its recommended application in seed treatment.

Materials and methods

Preparation of *Beejamrit*

The *Beejamrit* input was prepared as before (Bishnoi and Bhati 2017) with a minor modification by mixing the cow dung, the cow urine, and the lime at fixed quantities. This study collected cow dung & cow urine from indigenous Sahiwal cross-breed cattle grazing at the Narendrapur Ramakrishna Mission Ashrama, India. As per the Gurukul protocol, 5-times (e.g., 250 gm) of limestone were added per 20 L of the *Beejamrit* preparation compared to the earlier method (Bishoi and Bhati 2017). Then, the mixed components were enriched further with forest soils. Notably, the forest soils in the *Beejamrit* preparation are considered crucial for the enrichment with a microbial load. In this study, the forest soils were collected from Rajabhatkhawa forest village, situated in North Bengal of India (Latitude – 26.6496759°N; Longitude – 89.5617723°E; and Altitude – 221 m). The status of the organic carbon content, 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 solution was incubated further for different periods (*e.g.*, 0-day as control, 1-day, 2-days, 3-days, 4-days, 5-days, 6-days, and 7-days). The incubation study was carried out in a closed container under static conditions at room temperatures ranging from 25 to 30 °C. However, the formulation was stirred twice daily for proper aeration and uniform decomposition. Finally, the *Beejamrit* samples were collected after definite periods and passed through a fine muslin cloth to receive the final products. The composition of *Beejamrit*, in detail, is shown in Supplementary Table 2.

Estimation of organic carbon

The organic carbon content was determined by a standard method (Walkley and Black 1934). Briefly, the samples were first oxidized in 1 (N) potassium dichromate solution and concentrated sulphuric acid. Oxidized aqueous samples were titrated further against 0.5 (N) ferrous ammonium sulphates in the presence of ferroin as an indicator to score. The titrated value, thus generated, corresponds to oxidizable organic carbon. Finally, the titrated value was converted into oxidizable organic carbon by 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, protein extraction was performed in phosphate buffer (pH 7.0). The Folin ciocaltau reagent was then used for colorimetric analysis by the UV-Vis spectrophotometer at 660 nm wavelength.

Determination of the culturable microbial population

In order to determine the microbial diversity and their dynamics, the *Beejamrit* formulation collected after different days of decomposition was serially diluted in appropriate concentrations and was subsequently plated over specific selective nutrient agar media by following standard methods. The details of growth media and their cultural conditions for a diverse group of microbes are mentioned in Supplementary Table 3. Similarly, the plant beneficial bacteria, such as the free-living nitrogen fixers (FNFs) and the phosphate solubilizing bacteria (PSBs), were cultured and scored based on their colony-forming ability by following a standard method. It is noteworthy that the plates of 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 et al. 1954). The extraction of available P was performed on phosphorus-free activated charcoal dissolved in a 0.5 (M) sodium bicarbonate solution. Next, the filtrate samples were acid neutralized by adding 2.5 (N) sulphuric acids. The p-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 3-acetic acid (IAA)

The indole compounds of *Beejamrit* were assayed quantitatively, as reported before (Salkowski 1885). Briefly, 10 ml of the *Beejamrit* samples were taken into a falcon tube and centrifuged at 10,000 rpm for 10 min. Next, 2 ml supernatant of each sample was taken out carefully, followed by the addition of 2 drops of orthophosphoric acid. Then, 4 ml of the Salkowski's reagent (0.5 M FeCl₃ solution in 35% perchloric acid) was added to it in the dark and was incubated further at room temperature for 25 min. It is worth noting that the golden yellow colour of the final *Beejamrit* solution turns into different grades of pink colour based on the indole content after the addition of Salkowski's reagent. Finally, the colour intensity of each sample was measured at a 530 nm wavelength against a control (*e.g.*, sterile distilled water) and the standard concentrations of IAA.

The High-Performance Thin Layer Chromatography (HPTLC) technique, on the other hand, was used to quantify the IAA content of the *Beejamrit* solution (Goswami et al. 2015). The extractants were first isolated in ethyl acetate solvent and then placed onto the TLC plate and air-dried. Finally, the intensity was measured by scanning the TLC plate in absorbance-reflectance mode at a 256 nm wavelength.

Plant-based bioassays

The field experiment was conducted during the winter season of 2021–2022 at the Organic Farming Centre of Ramakrishna Mission Vivekananda Educational and Research Institute (Latitude – 22.439420°N; Longitude – 88.401792°E; and Altitude – 9 m). This plot has been maintained under organic nutrient management for the last six years. The vermicompost (*e.g.*, 9 t per ha) has been applied in this experiment, assuming the poor nutrient quality of *Beejamrit* and has no role as a nutrient source (Smith et al. 2020). The experiment consists of nine treatments (*e.g.*, the *Beejamrit* solutions after different periods and sterile

distilled water as a control), laid out in a randomized block design (RBD) with three replicates. The net plot size for each treatment was 4 m × 3 m. The seeds of the French bean (Variety: Falguni) were sown at 50 kg per ha at a spacing of 20 cm (row to row) × 15 cm (plant to plant). The initial soil physico-chemical and microbiological properties, in detail, can be found in Supplementary Table 4.

Three plantlets were randomly selected from each replicate for measurement of shoot length, root length, number of pods per plant, pod length, and pod weight. On the other hand, seed germination followed by their survival was monitored routinely up to 12-days after the shoot emergence in each block (e.g., n = 50). The seed germination and seedling survival, in percentages, were calculated as the number of seeds germinated over the total number of seeds sown and the number of seedlings survived over the total number of seeds germinated, respectively.

A plate-based assay was conducted similarly under laboratory conditions to study seed germination and root-shoot length for rice (Variety: Satabdi/IET4786), vegetable pea (Variety: MS10), and French beans (Variety: Falguni). The data were, finally, collected after 7-days of incubation.

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, whereas the bar indicates the 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>). For the agronomic data analysis, OPSTAT software was used as illustrated by Sheoran Programmer, Computer Section, CCSHAU, Hisar for Analysis of Variance (ANOVA) (<http://www.202.141.47.5/opstat/index.asp>).

Network analysis

The interrelationship among pH, organic carbon, total soluble protein, PSBs, FNFs, available P, and IAA was developed in visual representation using the graph-theoretic approach. The bivariate correlation matrix involving the said parameters was used as a symmetric adjacency matrix for generating the network diagram. The UCINET 6 software (Borgatti et al. 2002) develops the adjacency matrix, and NetDraw software (Borgatti 2002), on the other hand, constructs the weighted graph in this study.

Results

The gradual depletion of organic carbon and total soluble protein in the *Beejamrit* solution after its incubation

In this study, we observed that the organic carbon (in percent value) in the *Beejamrit* solution undergoes gradual depletion followed by its incubation (Fig. 1a). It is also evident from this study that the organic carbon depletion occurred more rapidly after 1-day of decomposition ($p < 0.0001$). On a similar note, we also observed that total soluble protein (mg per ml) in the *Beejamrit* solution decreases progressively after 1-day of incubation ($p < 0.0001$) (Fig. 1b), as previously observed in organic carbon decomposition (Fig. 1a). In conclusion, we report an amazingly uniform decomposition pattern of organic carbon and protein residues in the *Beejamrit* solution.

Decomposition leads to pH changes in the *Beejamrit* solution

We next sought to determine the pH of the *Beejamrit* solution collected after different incubation periods. The study showed a gradual drop in the pH of the *Beejamrit* solution after more days of incubation ($p < 0.0001$) (Fig. 1c). The initial alkaline pH of the *Beejamrit* solution (8.51 ± 0.03), on the other hand, indicates the presence of cow urine and limes in the formulation. We finally observed that the pH of the *Beejamrit* solution becomes neutral after 7-days of incubation. Following this trend, the pH of the *Beejamrit* solution approaches 7.49 ± 0.06 , which is close to physiological pH, after 4-days of decomposition (Fig. 1c). Together, we conclude that the pH of the *Beejamrit* solution ranges from slightly alkaline to neutral based on its different days of incubation.

Microbial population dynamics in *Beejamrit*

In this study, we observed that the overall bacterial population of the *Beejamrit* input does not increase considerably ($p > 0.05$) up to 2-days of initial decomposition (Fig. 2a). However, the bacterial population shows rapid multiplication after 3-days of incubation ($p < 0.001$) and approaches its highest number in terms of the colony-forming unit (CFU) after 5-days of incubation ($2.43 \pm 0.03 \times 10^8$ CFUs per ml). From 6-days onwards, the bacterial population is surprisingly showing a declining trend. These observations in the bacterial population nicely correlate with the decomposition pattern of organic matter in the *Beejamrit* solution (Fig. 1a and b). The actinomycetes and fungi population, on the other hand, do not change significantly up to 2- and

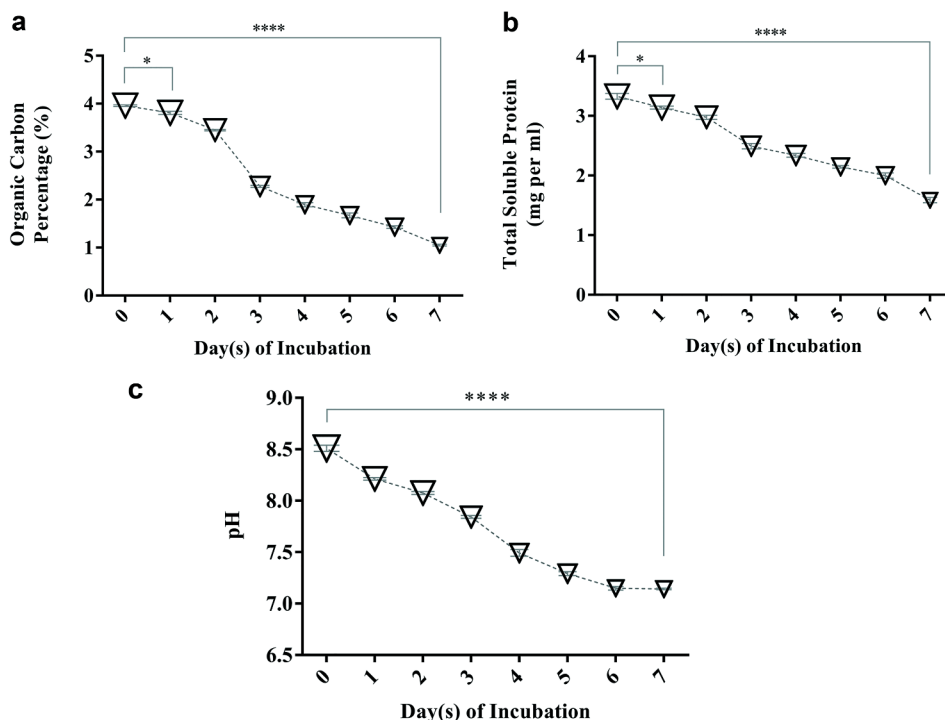
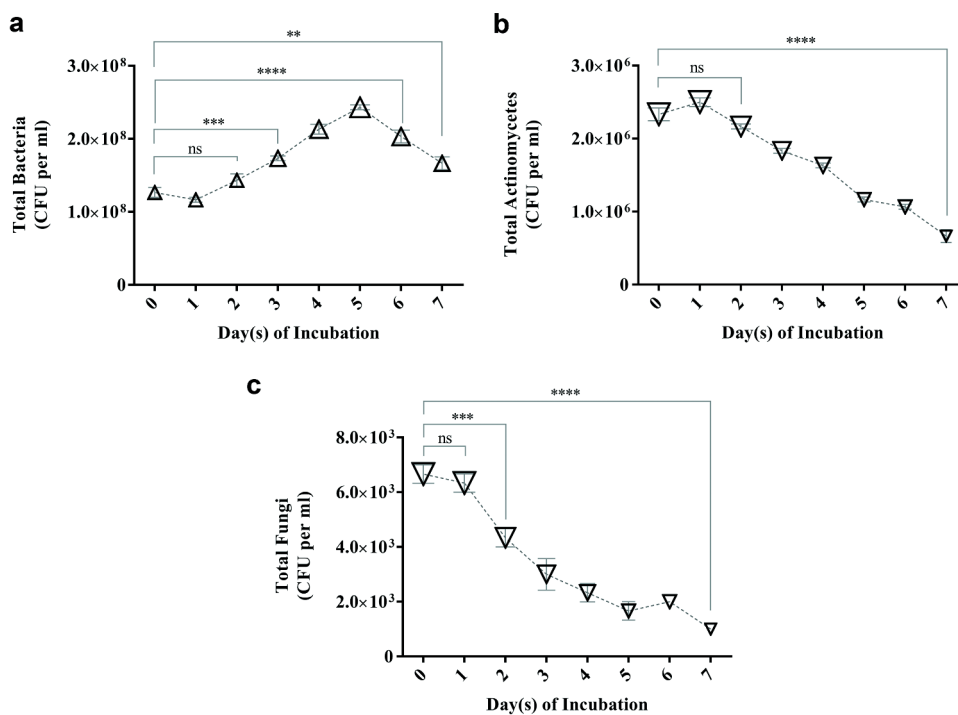


Fig. 1 The physico-chemical properties of the *Beejamrit* input indicate the gradual depletion of organic matters concurrent with pH changes after different incubation periods. The (a) organic carbon and (b) total soluble protein were analyzed using three replicates, and the bar indicates a standard 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 was quantified and expressed in milligrams per milliliter (mg/ml). (c) The pH of the *Beejamrit* solution decreases over the days of incubation. The y-axis denotes the mean value of three replicates with a standard error of the mean (SEM). Here, asterisks indicate significant differences between 0-day as control and different days of incubation based on Dunnett’s multiple comparisons tests. * $p < 0.05$; **** $p < 0.0001$

Fig. 2 The diversity and dynamics of the microbial population in *Beejamrit*. The total culturable (a) bacteria, (b) actinomycetes, and (c) fungi populations were counted and analyzed 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). Asterisks indicate significant differences between 0-day as control and different days of incubation based on Dunnett’s multiple comparisons tests. ‘ns’ non-significant; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$



1-day of incubation, respectively ($p > 0.05$). However, both these populations afterward decreased gradually upon further incubation ($p < 0.0001$) (Fig. 2b and c). In conclusion, this study establishes that the *Beejamrit* solution is a microbial formulation, and the microbial abundance of *Beejamrit* changes based on the days of its incubation.

Beejamrit is a potential source of plant beneficial bacteria

This study reveals that the population of free-living nitrogen fixers (FNFs) gradually grows over days of decomposition. However, their population increases substantially after

3-days of incubation ($p < 0.0001$) and reaches a maximum ($2.30 \pm 0.12 \times 10^7$ CFUs per ml) after 4-days of decomposition (Fig. 3a). We next sought to study phosphate solubilizing bacteria (PSBs) to elucidate their potential role in phosphorus availability to crop plants. Incidentally, we also observed in this study that the level of available phosphorus content in *Beejamrit* increases gradually and reaches its peak in concentration (151 ± 3.54 mg per ml) after 4-days of incubation (Supplementary Fig. 1). However, the plant-available form of phosphorus gets depleted at 5-days of decomposition and onwards. On a similar trend, we also observed here that the population of PSBs increases significantly in the *Beejamrit* input followed by its incubation

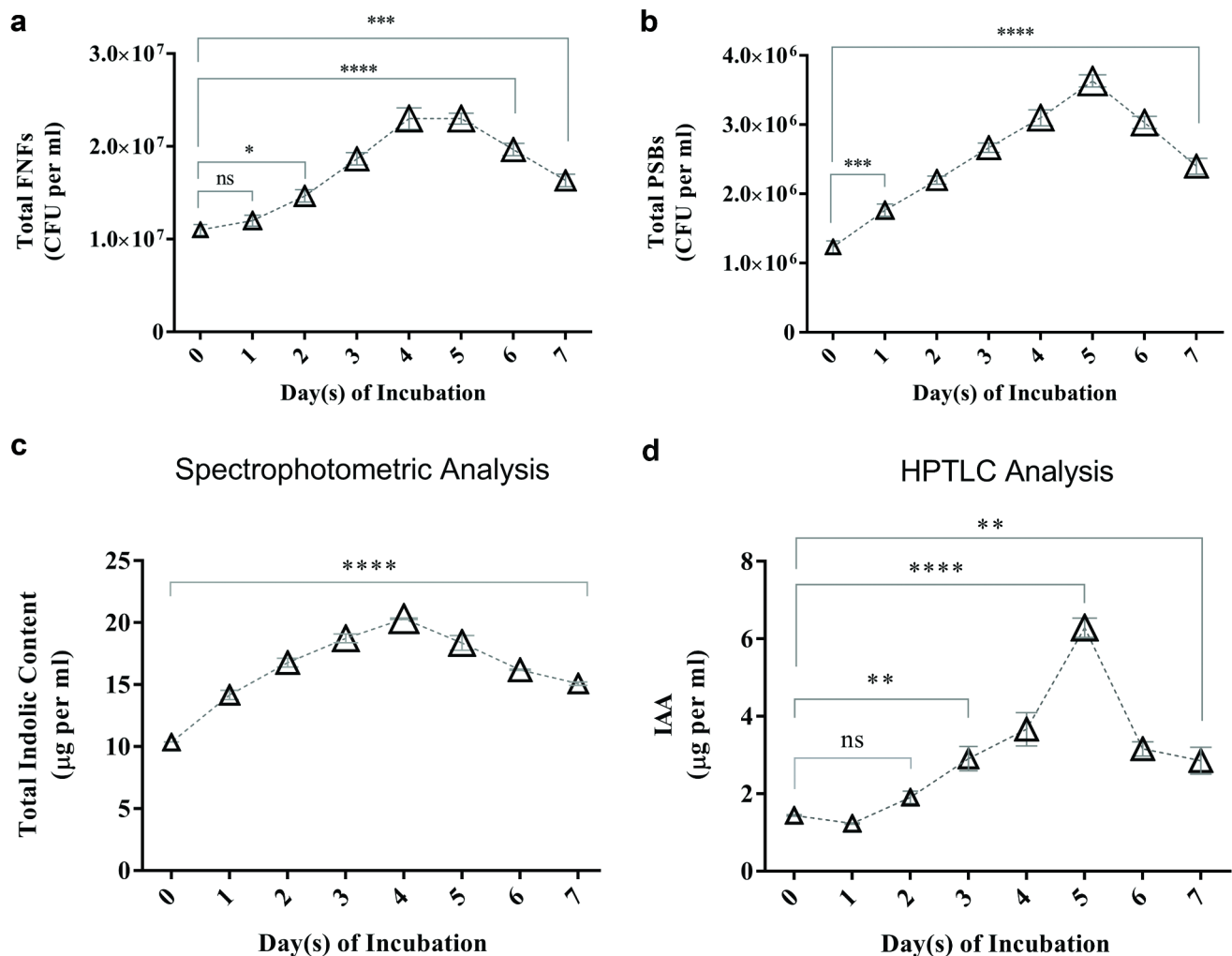


Fig. 3 *Beejamrit* acts as a potential source of plant biostimulant. (a–b) Dynamic plots of plant beneficial bacteria in the *Beejamrit* input. Here, the total culturable (a) free-living nitrogen fixers (FNFs) and (b) phosphate solubilizing bacteria (PSBs) populations were counted and examined with three replicates with a standard error of the mean (SEM). The y-axis denotes the population of microbes in CFU per milliliter (CFU/ml). (c–d) Determination of the IAA content of the *Beejamrit* solution by the (c) Spectrophotometric and (d) High-Performance Thin Layer Chromatography (HPTLC) techniques. The IAA content of *Beejamrit* solution, after different incubation periods, was determined with three replicates with a standard error of the mean (SEM). The y-axis denotes the IAA concentration in micrograms per milliliter (µg/ml). Asterisks indicate significant differences between 0-day as control and different days of incubation based on Dunnett's multiple comparisons tests. 'ns' non-significant; * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$

($p < 0.0001$), and it reaches a maximum ($3.63 \pm 0.09 \times 10^6$ CFUs per ml) after 5-days of incubation (Fig. 3b). This result strongly suggests that the population of PSBs has a direct role in the level of available phosphorus content in *Beejamrit*. Together, our results showed a dynamic structure of plant beneficial bacterial niche, indicating a peak of their population between 4-to-5-days after decomposition of the *Beejamrit* formulation (Fig. 3a and b). Overall, these findings suggest that the *Beejamrit* solution, at least after 4-days to till 5-days of incubation, would yield optimum benefits in terms of beneficial microbial population when applied to the crop seeds.

A dynamic landscape of the indolic class of plant growth regulators, including IAA, in *Beejamrit*

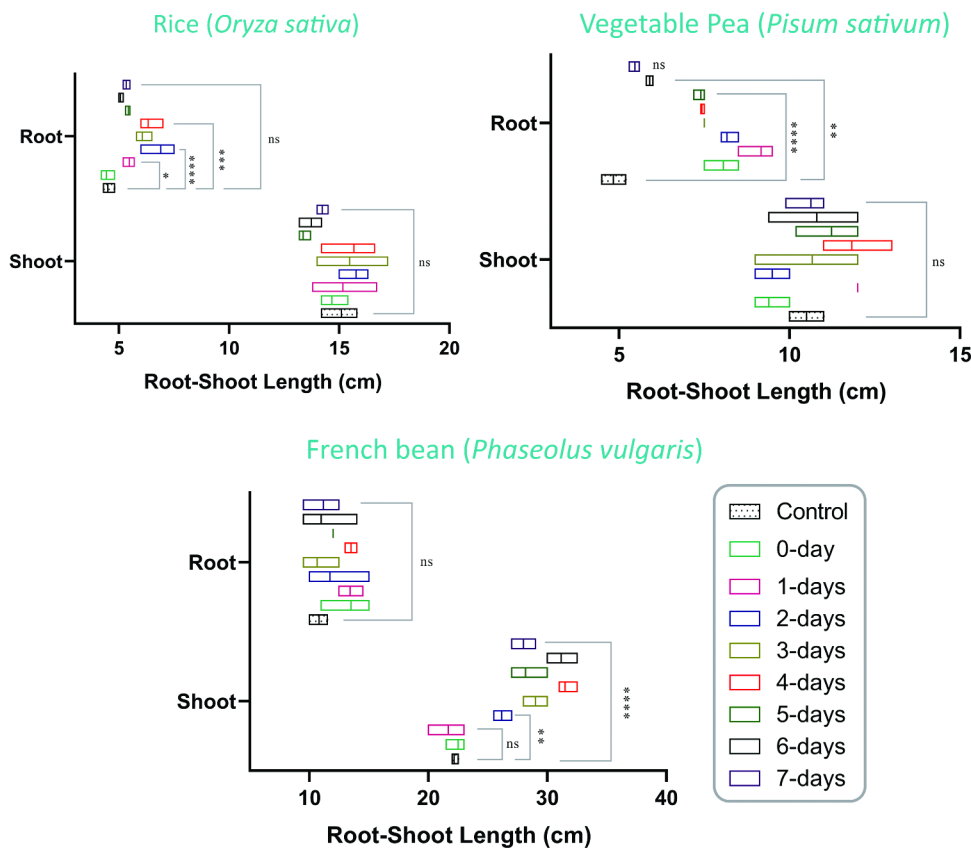
In this study, we observed that the IAA concentration in the *Beejamrit* solution rises gradually after days of incubation ($p < 0.0001$) (Fig. 3c). It is worth noting that the IAA content of *Beejamrit* solution reaches its highest concentration ($20.33 \pm 0.06 \mu\text{g per ml}$) after 4-days of decomposition (Fig. 3c). However, this spectrophotometric method is based on the reaction with all indolic derivatives and hence detects total indolic concentration rather than IAA more precisely. We, therefore, extended this study to measure the IAA dynamics specifically by the HPTLC technique. The

dynamic plot confirms that the *Beejamrit* solution is indeed a potential source of IAA ($1.20 > \text{IAA} < 6.75 \mu\text{g per ml}$) (Fig. 3d). The variation in the IAA data so far observed may reflect the sensitivity and specificity of two different techniques. In conclusion, this study establishes that the *Beejamrit* solution is a promising source of indolic compounds, including IAA.

A plant-based bioassay of the *Beejamrit* formulation reveals its potential application as a seed priming agent

The study on the *Beejamrit* solution in terms of its plant beneficial bacteria and IAA content prompted us to investigate further their role as a plant biostimulant in three crop seeds, namely rice (*Oryza sativa*), vegetable pea (*Pisum sativum*), and French bean (*Phaseolus vulgaris*). In the lab-based experiments, we did not observe a significant effect of *Beejamrit* on seed germination in these plant species, except in the case of the vegetable peas (Supplementary Table 5). However, the coating of *Beejamrit* had a positive impact on the root-shoot length in all three species (Fig. 4). The root length was significantly higher in the *Beejamrit* coated seed samples (e.g., the *Beejamrit* formulation prepared at least after 1-to-5-days of incubation) in rice and vegetable peas, whereas there is no effect on shoot length ($p > 0.05$) in these

Fig. 4 The effect of the *Beejamrit* solution on the root-shoot trait of crop plants. The data on root-shoot length of (a) rice, (b) vegetable pea, and (c) French beans, grown under laboratory conditions, were analyzed using three replicates with a standard error of the mean (SEM). Here, asterisks indicate significant differences between 0-day as control and different days of incubation based on Dunnett’s multiple comparisons tests. ‘ns’ non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$



plant species. We, however, observed the opposite trend in the case of French beans, reflecting a significantly higher shoot length in most of the *Beejamrit* treatments with no apparent difference ($p > 0.05$) in root length (Fig. 4). Our study, together, indicates a positive effect on root-shoot length trait in the *Beejamrit*-treated seed samples in three different crop plants under laboratory conditions.

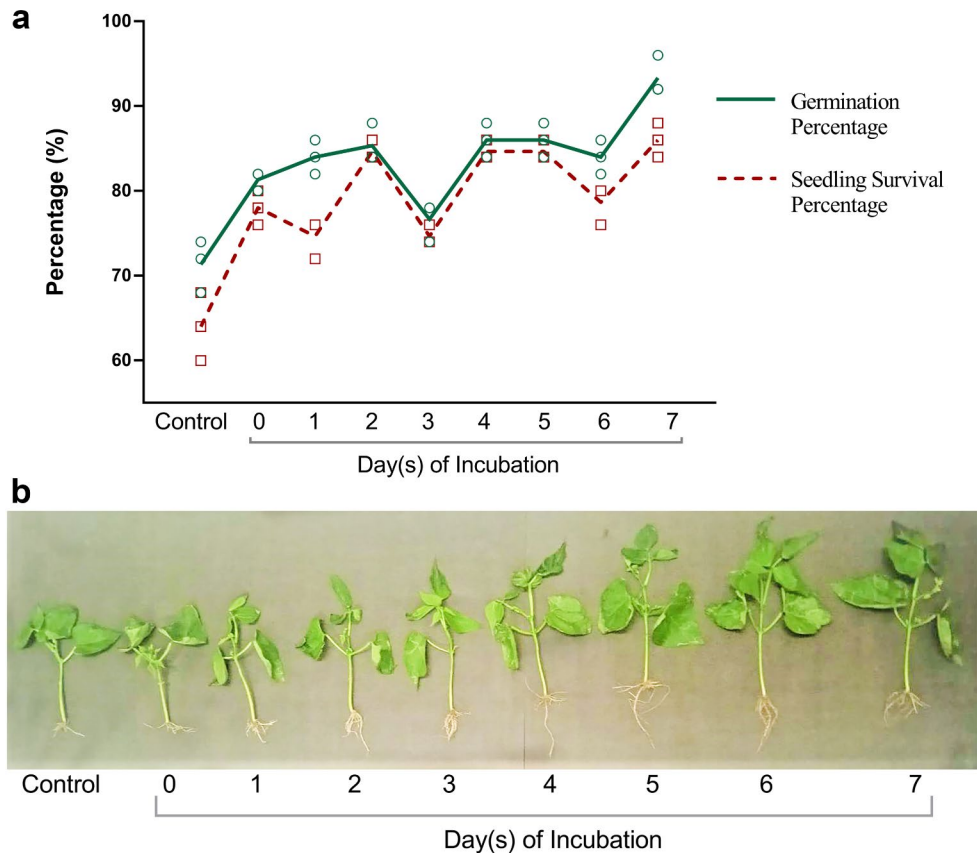
To establish the effect of the *Beejamrit* formulation further, we next extended this study to the field conditions under organic nutrient management during the winter season. In the field experiment, the *Beejamrit*-treated seed samples showed significantly higher seed germination followed by a higher seedling survival rate ($p < 0.01$) after 12-days of shoot emergence (Fig. 5a; Supplementary Table 6). These results suggest that the *Beejamrit* solution has a definite impact on seed germination traits under field conditions ($p < 0.01$). The uniform germination rate of about 86-to-95% observed in French beans under laboratory conditions, on the other hand, establishes higher seed viability in French beans (Supplementary Table 5). In this context, the field data on seed germination strongly indicates that the *Beejamrit* solution may ameliorate the negative inhibition and help the seed germination and initial seedling survival in the soil conditions. In addition, the shoot length, consistent with our observation in the laboratory conditions, was significantly higher ($p < 0.01$) in the *Beejamrit*-coated

samples (Fig. 5b; and Supplementary Table 6). However, we did not observe significant changes ($p > 0.05$) in other agronomic traits of French beans, except for the pod length ($p < 0.01$) (Supplementary Table 6). This result may indicate no apparent additive effect of *Beejamrit* on yield attributes, reflecting its poor nutrient content. In addition, there was no discerning trend observed for other plant attributes over different periods of incubation, as in the case of *Beejamrit* (Supplementary Table 6). Together, our results establish that the *Beejamrit* formulation is an ideal organic source for seed treatment and helps to improve seed germination and seedling survival rate under field conditions.

Discussion

In this work, we aimed to study the microbial composition of the *Beejamrit* formulation and its microbial dynamics over 7-consecutive days of its incubation. To elucidate microbial dynamics, we were initially interested in analyzing the content of the organic carbon and total soluble protein (i.e., nutrient sources) of the *Beejamrit* solutions collected after different days of incubation. In this study, we observed that the concentration of organic carbon and soluble protein, collectively the carbon and nitrogen sources of *Beejamrit*, depletes rapidly after 2-days of incubation (Fig. 1a and b).

Fig. 5 The efficacy of *Beejamrit* as a seed priming agent on French bean seedlings. **(a)** The germination and seedling survival of French beans grown in organic fields were measured in three replicates after 12-days of shoot emergence and represented as a percentage value. Here, the dark blue-green circles denote the data of germinated seedlings, whereas the red-pink coloured rectangular boxes represent the number of seedlings that survived after 12-days of their shoot emergence. **(b)** The seedling morphology of French beans. The seedlings were either treated with the *Beejamrit* solution after different incubation periods or sterile distilled water as a control. The image was finally taken 15-days after the first shoot emergence.

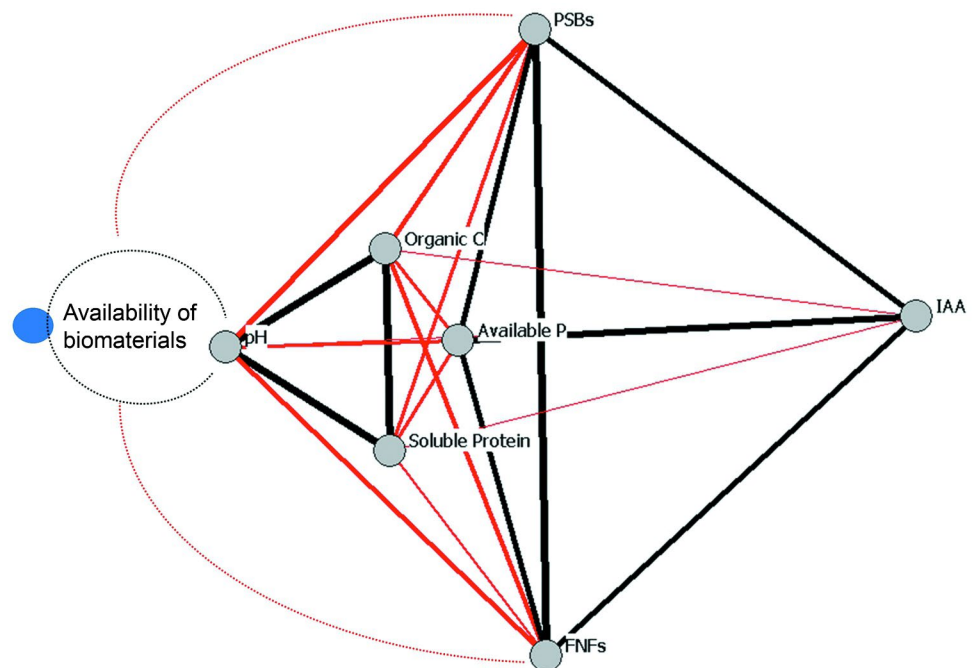


Our result strongly indicates the decomposition of organic matter into simple forms of biomolecules in the system and may subsequently get emitted either as CO_2 and CH_4 in the case of organic carbon (Grandy and Neff 2008; Berg 2000) or as NH_3 , N_2O , and NO_2 from the system (Körner 2007). Microbes are ubiquitously present in any ecological niche and can play a significant role in carbon and nitrogen cycling (Lennon and Jones 2011). We report in this study that the bacterial population grows faster in the *Beejamrit* solution after 3-days of incubation (Fig. 2a). However, the bacterial population also shows a decline after 5-days of decomposition. On a similar trend, we also observed that the decomposition rate of organic matters in *Beejamrit* afterward becomes saturated gradually (Fig. 1a and b). These results, together, indicate that the *Beejamrit* preparation cannot provide nutrient support for the microbial multiplication over a longer time, at least after 5-days of incubation. The populations of actinomycetes and fungi, on the other hand, started to fall steadily from the initial day of decomposition (Fig. 2b and c). 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). These observations strongly support its application as an organic formulation to combat seed-borne diseases, primarily due to fungal pathogens.

In the case of any biological system, organic carbon and proteins are 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 governs, in particular, the rate of its decomposition (Ashraf et al. 2020; Zhao et al. 2018). 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, pH, on the other hand, is also known to regulate the availability of nutrients (Jin and Kirk 2018). Hence, there may be a dynamic network between the pH and the nutrient availability that subsequently influences microbial communities. From these points of reference, we propose that the decomposition of complex biomolecules leads to pH changes in the *Beejamrit* solution during the initial phase of microbial actions. Earlier 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 in *Beejamrit* (i.e., possibly 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). The rapid growth of phosphate solubilizing bacteria (PSBs), in consequence, degrades complex carbon molecules and generates readily energy-releasing forms of sugar. This degradation process may, in turn, compensate for the energy required for a thermodynamically unfavorable nitrogen-fixing reaction (Rojas et al. 2001). Importantly, it was shown earlier that the depletion of nitrogen availability in combination with the enrichment of various carbon forms such as sucrose, malate, and mannitol promotes the growth and multiplication of the free-living nitrogen fixers (FNFs) in the rhizosphere (Smercina et al. 2019). In addition, the activity of phosphate solubilizing

Fig. 6 An 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 relationships.



bacteria (PSBs) may cause a release of available phosphorus that influences nitrogen fixation positively (Smircina et al. 2019). In this microbe-based network, the niche system accumulates a range of mild acids subsequently during biological nitrogen fixation reactions and may indirectly promote the mineral phosphate solubilization and subsequent 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. 3a and b). This study also reports a depletion of soluble protein content in the *Beejamrit* solution (Fig. 1b). This observation may indicate that proteins get converted into simpler amino acids intermittently and is likely to supply tryptophan, one of the twenty natural amino acids, to the *Beejamrit* system. Growing evidence further establishes a direct link between tryptophan and the microbial mode of IAA production (Ahmad et al. 2008; Saharan and Nehra 2011). The free-living nitrogen fixers (FNFs) and the phosphate solubilizing bacteria (PSBs), in fact, are also competent in producing IAA from a well-known precursor (e.g., tryptophan) *in-vivo*. Therefore, it would be fascinating to study how this dynamic *Beejamrit* system modulates the physico-chemical and microbiological properties after different days of incubation. To shed further light on this intriguing question, we propose a dynamic network of pH, organic carbon, soluble protein content, available phosphorus content, free-living nitrogen fixers (FNFs), and the phosphate solubilizing bacteria (PSBs), and how it may influence the microbial niche and the IAA content of the *Beejamrit* formulation (Fig. 6 and Supplementary Table 7).

To generate a model of this dynamic microbe-based network (Fig. 6), we followed the correlation-based network analysis, where the bivariate correlation coefficient is used as a measure (weight) of the dyadic relationship (the edge) (Batushansky et al. 2016; Toubiana et al. 2016, 2020; Matchado et al. 2021). Here, the elements in a complex biological system are conceptualized as vertices and their relationships as edges. In the last decade, a similar approach to understanding the system dynamics in a holistic view, especially of a complex biological system, is an emerging concept. In contrast, simple correlation studies can only trace a discrete bivariate relationship between two entities within a complex system. Where such reductionist assessments can describe the dyadic relationship between two elements, they are inadequate to understand the system dynamics within which the dyadic relationship occurs. The network model, thus generated in this study based on the correlation value

between element pairs, can assess the characteristics of system networks (density, centralization, etc.) and identify the central elements in the system, as in the case of the *Beejamrit* input (Fig. 6). This kind of network model, together with the high-throughput microbiome data of the *Beejamrit* system, can provide further insights into the greater detail of such a complex microbial system. Hence, we anticipate that this study will further stimulate comprehensive and high-throughput microbiome investigations in the future.

According to traditional knowledge, the most common practice among the Indian farming community is to use overnight *Beejamrit* preparation to receive optimum benefits. However, there is a lack of scientific reports or evidence to support such 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- and 5-days of incubation, respectively (Fig. 3a and b). Such a trend in plant beneficial bacterial dynamics correlates well with the highest observed IAA concentration after 4-to-5-days of decomposition (Fig. 3c and d). Our analyses, together, indicate that *Beejamrit* is the most effective based on its plant beneficial bacteria dynamics and the IAA content in *Beejamrit* at least after 4-days of incubation (Fig. 3). It is well known that indolic compounds help to promote cell division and plant cell elongation and hence, regulate plant growth (Uggla et al. 1996). The positive impact on root-shoot length further supports the role of *Beejamrit* as a source of plant growth regulators, and the data is consistent with other known seed priming agents reported earlier in rice, wheat, sorghum (Chen et al. 2021; Farooq et al. 2007; Ghobadi et al. 2012), and French beans (Gowthamchand et al. 2019). These results, together, indicate that *Beejamrit* as a microbial formulation may trigger the metabolic process of crop seeds that takes place during germination to seedling emergence. It may also act as a stimulus and provide buffering ability to alleviate the negative variability in agricultural field conditions. Our study, in support, reports the significantly better performance on seed germination, seedling survival, shoot length, and pod length of French bean treated with *Beejamrit* prepared at least after 4-to-5-days of incubation compared to the overnight formulation (Supplementary Table 6). These results nicely coincide with the plant beneficial microbes and IAA dynamics in the *Beejamrit* formulation after different days of incubation (Fig. 3). Based on correlation statistics, our data further suggest that the microbes of the *Beejamrit* formulation, delineating their role in IAA production and plant beneficial network, may influence directly or indirectly an overall improvement in seedling survival rate and shoot length trait in French beans (Supplementary Table 8). We assume that the overall improvement in the seed germination percentage followed by a higher seedling survival rate

in the *Beejamrit*-treated samples can have a cumulative impact on the French bean population in agricultural fields. The positive effect of *Beejamrit* on the germination percentage, seedling survival rate, and better pod length traits in French beans may further influence the total yield and the quality attributes of the crop, owing to higher economic profits for the farmers. Overall, we establish *Beejamrit* as a potential application as a seed coating cum priming agent in agriculture and can be an excellent mode of beneficial microbe delivery.

Conclusions

This study reports *Beejamrit* as a microbial formulation of plant beneficial bacteria, including the free-living nitrogen fixers (FNFs) and the phosphate solubilizing bacteria (PSBs). In addition, we also find that *Beejamrit* is a potent source of IAA, which is a well-known plant growth regulator. This study, in turn, suggests that *Beejamrit* can be used effectively as a plant biostimulant to promote plant growth and development. The *Beejamrit* input, in support, showed a significant effect on seed germination, root-shoot trait, and pod length in French beans. Overall, this finding recommends the application of *Beejamrit* in the seed treatment to improve the germination and initial seedling survival rate. In conclusion, our study provides scientific insight into the *Beejamrit* preparation, its dynamic plant beneficial microbial network, and its role as a seed priming agent.

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Conceptualization: SM, NR & GC.

Investigation and Data Generation: SM & SS.

HPTLC Analysis: SM, RB, BP & GC.

Plant-based Bioassay: SM, AC, KR & GC.

Statistical Analysis: MNA & GC.

Network Analysis: RG.

Funding Acquisition: NR & GC.

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Writing – Reviewing & Editing: SM, SS, MNA, RG, AC, KR, RB, BP, NR & GC.

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Ethics declarations

Conflict of interest Authors declare no competing or conflict of interest to declare that are relevant to the content of this article.

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References

- Ahemad M, Khan MS (2011) Functional aspects of plant growth promoting rhizobacteria: recent advancement. *Insight Microbiol* 1(3):39–54. <https://doi.org/10.5567/IMICRO-1K.2011.39.54>
- Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiol Res* 163(2):173–181. <https://doi.org/10.1016/j.micres.2006.04.001>
- Ashraf MN, Hu C, Wu L, Duan Y, Zhang W, Aziz T, Cai A, Abrar MM, Xu M (2020) Soil and microbial biomass stoichiometry regulate soil organic carbon and nitrogen mineralization in rice-wheat rotation subjected to long-term fertilization. *J Soils Sediments* 20(3):103–113. <https://doi.org/10.1007/s11368-020-02642-y>
- Batushansky A, Toubiana D, Fait A (2016) Correlation-based network generation, visualization, and analysis as a powerful tool in biological studies: a case study in cancer cell metabolism. *BioMed Res Intl Article ID* 8313272. <https://doi.org/10.1155/2016/8313272>
- Berg B (2000) Litter decomposition and organic matter turnover in northern forest soils. *For Ecol Manag* 133:(1–2):13–22. [https://doi.org/10.1016/S0378-1127\(99\)00294-7](https://doi.org/10.1016/S0378-1127(99)00294-7)
- Bharuchaa ZP, Mitjans SB, Pretty J (2020) Towards redesign at scale through zero budget natural farming in Andhra Pradesh, India. *Int J Agril Sustainability* 18(1):1–20. <https://doi.org/10.1080/14735903.2019.1694465>
- Bishnoi R, Bhati A (2017) An overview: Zero Budget Natural Farming. *Trends Biosci* 10(46):9314–9316. <https://doi.org/10.13140/RG.2.2.17990.83522>
- Borgatti SP, Everett MG, Freeman LC (2002) Ucinet 6 for Windows: Software for social network analysis. Analytic Technologies, Harvard, MA
- Borgatti SP (2002) Netdraw network visualization. Analytic Technologies, Harvard, MA
- Chadha S, Rameshwar, Ashlesha, Saini JP, Paul YS (2012) Vedic Krishi: Sustainable livelihood option for small and marginal farmers. *Indian J Tradit Know* 11(3):480–486
- Chen X, Zhang R, Xing Y, Jiang B, Li B, Xu X, Zhou Y (2021) The efficacy of different seed priming agents for promoting sorghum

- germination under salt stress. *PLoS ONE* 16(1):e0245505. <https://doi.org/10.1371/journal.pone.0245505>
- Devakumar N, Shubha S, Gouder SB, Rao GGE (2014) Microbial analytical studies of traditional organic preparations beejamrutha and jeevamrutha. *Building Org Bridges* 2:639–642
- Farooq M, Basra SMA, Khan MB (2007) Seed priming improves growth of nursery seedlings and yield of transplanted rice. *Archives Agron Soil Sci* 53(3):315–326. <https://doi.org/10.1080/03650340701226166>
- Ghobadi M, Abnavi MS, Honarmand SJ, Ghobadi ME, Mohammadi GR (2012) Effect of hormonal priming (GA3) and osmopriming on behavior of seed germination in wheat (*Triticum aestivum* L.). *J Agri Sci* 4(9):244–250. doi:<https://doi.org/10.5539/jas.v4n9p244>
- Grandy AS, Neff JC (2008) Molecular C dynamics downstream: the biochemical decomposition sequence and its impact on soil organic matter structure and function. *Sci Total Environ* 404(2–3):297–307. <https://doi.org/10.1016/j.scitotenv.2007.11.013>
- Goswami D, Thakker JN, Dhandhukia PC (2015) Simultaneous detection and quantification of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) produced by rhizobacteria from l-tryptophan (Trp) using HPTLC. *J Microbiol Methods* 110:7–14. <https://doi.org/10.1016/j.mimet.2015.01.001>
- Gowthamchand NJ, Ganapathi, Soumya TM (2019) Effect of bulky manures and fermented liquid organics on growth, yield, nutrient uptake and economics of French bean (*Phaseolus vulgaris* L.) under rainfed condition. *Intl J Agri Environ Biotechnol* 12(4):361–368. doi: <https://doi.org/10.30954/0974-1712.12.2019.10>
- Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann Microbiol* 60:579–598. <https://doi.org/10.1007/s13213-010-0117-1>
- Jin Q, Kirk MF (2018) pH as a primary control in environmental microbiology: 1. Thermodynamic Perspective. *Front Environ Sci* 6(21):1–15. <https://doi.org/10.3389/fenvs.2018.00101>
- Körner I (2007) Investigations into ammonia emissions during composting. In: Monteny GJ, Hartung E (eds) *Ammonia emissions in agriculture*. Wageningen Academic Publishers, pp 221–223
- Lennon JT, Jones SE (2011) Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nat Rev Microbiol* 9:119–130. <https://doi.org/10.1038/nrmicro2504>
- Matchado MS, Lauber M, Reitmeier S, Kacprowski T, Baumbach J, Haller D, List M (2021) Network analysis methods for studying microbial communities: A mini review. *Comput Struct Biotechnol J* 19:2687–2698. <https://doi.org/10.1016/j.csbj.2021.05.001>
- Olsen SR, Cole CV, Watanabe FS, Dean LA (1954) Estimation of available phosphorus in soils by extraction with sodium bicarbonate. Washington, DC: US Department of Agriculture 939:19
- Patel SK, Sharma A, Singh GS (2020) Traditional agricultural practices in India: an approach for environmental sustainability and food security. *Energ Ecol Environ* 5(4):253–271. <https://doi.org/10.1007/s40974-020-00158-2>
- Rojas A, Holguin G, Glick BR, Bashan Y (2001) Synergism between *Phyllobacterium* sp.(N₂-fixer) and *Bacillus licheniformis* (P-solubilizer), both from a semiarid mangrove rhizosphere. *FEMS Microbiol Ecol* 35(2):181–187. <https://doi.org/10.1111/j.1574-6941.2001.tb00802.x>
- Saharan BS, Nehra V (2011) Plant growth promoting rhizobacteria: a critical review. *Life Sci Med Res* 21:1–30
- Salkowski E (1885) Über das Verhalten der skatolcarbonsäure im Organismus. *Z Physiol Chem* 9:23–33
- Sharma IP, Kanta C, Dwivedi T, Rani R (2020) Indigenous agricultural practices: A supreme key to maintaining biodiversity. In: Goel R, Soni R, Suyal D (eds) *Microbiological advancements for higher altitude agro-ecosystems & sustainability*. Rhizosphere Biology. Springer, Singapore. https://doi.org/10.1007/978-981-15-1902-4_6
- Sharma SK, Jain D, Choudharya R, Jat G, Jain P, Bhojiya AA, Jain R, Yadav SK (2021) Microbiological and enzymatic properties of diverse *Jaivik Krishi* inputs used in organic farming. *Indian J Tradit Know* 20(1):237–243
- Smercina DN, Evans SE, Friesen ML, Tiemann LK (2019) To fix or not to fix: controls on free-living nitrogen fixation in the rhizosphere. *Appl Environ Microbiol* 85:e02546–e02518. <https://doi.org/10.1128/AEM.02546-18>
- Sreenivasa MN, Naik N, Bhat SN (2009) Beejamrutha: A source for beneficial bacteria. *Karnataka J Agric Sci* 22(5):1038–1040
- Toubiana D, Xue W, Zhang N, Kremling K, Gur A, Pilosof S, Gibon Y, Stitt M, Buckler ES, Fernie AR, Fait A (2016) Correlation-based network analysis of metabolite and enzyme profiles reveals a role of citrate biosynthesis in modulating N and C metabolism in *Zea mays*. *Front Plant Sci* 7:1022. <https://doi.org/10.3389/fpls.2016.01022>
- Toubiana D, Sade N, Liu L, Rubio Wilhelmi MDM, Brotman Y, Luzarowska U, Vogel JP, Blumwald E (2020) Correlation-based network analysis combined with machine learning techniques highlight the role of the GABA shunt in *Brachypodium sylvaticum* freezing tolerance. *Sci Rep* 10(1):116. <https://doi.org/10.1038/s41598-020-61081-4>
- Uggla C, Moritz T, Sandberg G, Sundberg B (1996) Auxin as a positional signal in pattern formation in plants. *Proc Natl Acad Sci USA* 93(17):9282–9286. <https://doi.org/10.1073/pnas.93.17.9282>
- Walkley A, Black IA (1934) An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method. *Soil Sci* 37(1):29–38
- Waterborg JH (2009) The Lowry method for protein quantitation. In: *The Protein Protocols Handbook Humana Press*, Totowa, NJ, pp, 7–10
- Weintraub MN, Schimel JP (2003) Interactions between carbon and nitrogen mineralization and soil organic matter chemistry in arctic tundra soils. *Ecosystems* 6(2):0129–0143. <https://doi.org/10.1007/s10021-002-0124-6>
- Zhao FZ, Ren CJ, Han XH, Yang GH, Wang J, Doughty R (2018) Changes of soil microbial and enzyme activities are linked to soil C, N and P stoichiometry in afforested ecosystems. *For Ecol Manag* 427(1):289–295. <https://doi.org/10.1016/j.foreco.2018.06.011>
- Smith J, Yeluripati J, Smith P, Nayak DR (2020) Potential yield challenges to scale-up zero budget natural farming. *Nat Sustain* 3(3):247–252. <https://doi.org/10.1038/s41893-019-0469-x>

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