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Metabolic analysis of liquid formulations of organic manures and its influence on growth and yield of *Solanum lycopersicum L.* (tomato) crop in field

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ABSTRACT

We report biochemical analysis of two formulations of liquid Organic Manures (OMs), which were prepared using raw materials readily available to farmers in fields; thus presenting them with cost-effective organic preparations. Previously reported microbial analysis of these OMs indicated presence of rich microbial consortia which enhance plant growth by making nutrient available to crop from soil. Likewise, we observed stimulatory effects of these two OMs on tomato plants in field trials that were marked by improved seed germination and growth by 2-and 3-folds, respectively. Metabolic profiling of the two preparations using Nuclear Magnetic Resonance (NMR) spectroscopy revealed presence of several plant-growth regulators, nucleobases, vitamins, amino acids, sugars and organic acids. This study provides a vital biochemical link in understanding the positive effect of organic molecules of microbial origin in OMs. But, along with these biomolecules, we also observed traces of fungicides and herbicides in all the preparations hinting at contaminated raw materials. This highlights the importance of robust analysis procedures in animal feeds and plant materials using analytical tools such as NMR spectroscopy based analysis that will aid farmers in not only preparing high quality nutrient management systems but with minimal residual pesticides. Moreover, cost effective preparations of OMs from animal wastes will open a window for organic farming beneficial for human society.

1. Introduction

Soil is an essential component of our ecosystem that influences several important biological processes for plant such as providing a substratum and various nutrients for their growth and development. Hence, it is necessary to maintain healthy soil conditions. Post green-revolution, usage of chemical fertilizers involved single or combinations of nitrogen, phosphorus and potassium (N, P and K) components in the fertilizers have hampered soil health by reducing organic content, increasing salinity, disturbing local pH (Wang et al., 2008) leading to disruption of microbial populations that support crop growth. Therefore, it is pertinent to employ eco-friendly agricultural practices for sustainable food production like biofertilizers (Kamilova et al., 2006a, 2006b; Lugtenberg and Kamilova, 2009), endophytes (Verma et al., 2001; Hammer and Rillig, 2011; Zhang et al., 2015) and organic farming (Heeb et al. 2006; Oliveira et al., 2013; Tonfack, 2013) to improve soil health.

Solanum lycopersicum L. (tomato) is one of the important cash crops grown throughout the world and is the most widely used processed crop in several condiments. It is widely grown in several states in India due to its relative ease in production, yet farmers face increasingly poor yield (Tiwari et al., 2006) due to application of chemical fertilizers in fields. But the cost of inorganic fertilizers is continuously on rise, especially due to fluctuation in petroleum prices, which is required for urea production. Hence using cost-effective alternatives in Organic Manures (OM) is the need of hour. Since tomato represents a valuable commercial crop and has been widely studied as a model system (Heeb et al., 2006; Reeve and Drost, 2012; Tonfack et al., 2013; Benard et al., 2015), with a short life of three months. It was selected as a test crop to study the effects of two popular OMs viz. Panchagavya (*Pc*)- (Ali et al., 2011; Gore and Sreenivasan, 2011) and Jeevamrutha (*Ja*)- (Gore and Sreenivasan, 2011), which are prepared from cattle waste and other readily available materials. Microbiological analysis of the

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OMs have revealed presence of mixed microbial consortia (Gore and Sreenivasan, 2011) containing N_2 fixers, phosphate solubilizers, lignin and cellulose degraders etc. The use of these liquid OMs have resulted in good yield and quality of crops. While the plant growth promoting factors have been extensively investigated, the exact nature of the organics in these formulations is unknown.

Nuclear Magnetic Resonance (NMR) spectroscopy is an important non-destructive tool that is routinely employed in metabolomics (Coen et al., 2008; Ulrich et al., 2008; Smolinska et al., 2012; Halouska et al., 2013), which provides invaluable information on various cellular processes. In recent years, metabolomics database has witnessed an increase in library of organic molecules and thereby identification of metabolic pathways will provide a deeper understanding of the benefits of OM in increasing the crop yield. Currently, it is a cost-effective tool in performing metabolomic studies on a wide variety of complex systems. Herein, we employed NMR spectroscopy based metabolomic fingerprinting approach to identify various biochemicals that make these OMs rich in nutrients that support plant growth and fruiting.

2. Materials and methods

2.1. Materials

Tomato seeds (*Solanum lycopersicum L.*, F1-Hybrid-250) were purchased from Sarpan Hybrid seeds Dharwad, Karnataka, India. Analytical grade D_2O (99.90%, Department of atomic energy, Government of India) and $CDCl_3$ (99.80%, Euriso-top, France) were used for the NMR experiments. Other materials, which are readily available such as milk, curd, clarified butter, jaggery, palm nectar, gram flour and banana were purchased from the local market. Cow dung and urine was purchased from the local farm yard.

2.2. Fertilizer preparations and application

Panchagavya (*Pc*) (Ali et al., 2011; Gore and Sreenivasan, 2011) was prepared using cow products like 5 kg dung, 0.5 kg clarified butter, 3 l urine, 2 l milk, 2 l curd, 3 l palm nectar and 1 dozen banana. Jeevamrut (*Ja*) (Gore and Sreenivasan, 2011) preparation included 3 l cow urine, 3 kg cow dung, 0.5 kg jaggery, 0.5 kg gram flour, 5 g of top soil from local garden and 13 l of water. All solid components for preparation of Pc and Ja were homogenized using household kitchen mixer (Bajaj GX8, 18 K rpm) before applying it to the cow dung which was stored in a 50 l plastic bucket. The mixture was rigorously mixed manually to prevent formation of clumps. Thereafter, the two liquid OMs were mixed daily twice for 10–15 min and covered with cotton cloth for a week before use. Prior to application 70 ml and 3 ml of Ja and Pc was diluted in 1 l tap water, respectively. Every week, 500 ml of the diluted OMs were applied manually by drenching.

2.3. Field trial

The experiment was conducted in 3 plots, each of 5×1 m^2 area, where tomato seeds were sowed in two bedding rows with a distance of 30×30 cm between seeds. Plants were watered once daily by gravity flow for 10 min during spring months of February-March and twice during summer season during the summer months of March-May. Biometric analysis such as seed germination rate, shoot length and fruit yield, were performed to study the effect of OMs. All data were compiled and analysed using mathematical tool Graphpad Prism 5.0 (GraphPad Software, San Diego, CA).

2.4. NMR spectroscopy

Five ml of each OM were dried in hot air oven maintained at 328 K (55 °C) for three days. To characterize lipophilic and hydrophilic extracts of the sample, the dried preparation was suspended in $CDCl_3$ and D_2O, respectively. All samples were measured on a Bruker NMR spectrometer (Bruker Biospin) with proton (^{1}H)-frequency of 500 MHz equipped with a 5 mm probe at constant temperature of 298 K (25 °C) applying Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence (Carr and Purcell, 1954). The NMR lock signal was obtained by adding D_2O and $CDCl_3$. ^{1}H NMR spectra were calibrated with respect to water and chloroform chemical shifts at 4.8 and 7.11 ppm, respectively. The spectra were visualized and processed using TopSpin NMR software (version3.2, Bruker Biospin Ltd.).

2.5. Identification of metabolites

^{1}H NMR spectrum contains information about the effective hydrophilic and hydrophobic metabolites present in the sample, which were elucidated using Biological Magnetic Resonance Data Bank (Ulrich et al., 2008) (BMRB-http://www.bmrb.wisc.edu/metabolomics/) metabolomics webserver. The NMR chemical shift peak lists were submitted to the database and a list of organic compounds that may occur in the sample were indexed with a score. Higher score reflected greater peak match between recorded spectra and chemical shifts in database. Analysis of metabolites for pathway and visualization was carried out using MetaboAnalyst webserver (Xia et al., 2015) (http://www.metaboanalyst.ca/).

3. Results

Our results highlight the effect of organic manures (OMs) on germination, shoot length, plant growth and fruiting. We observed 100% and 70% (100% by 4th week) germination (Table 1 and Fig. 1) of tomato seeds in 12 days, fertilized with *Pc* and *Ja* respectively. In stark contrast, untreated seeds showed only 50% germination. Further, we measured shoot length and fruit yield as biometric observations to test the efficacy of the OMs. Seventy days after sowing (DAS), we observed flowering of OM treated plants and by the 80th DAS we also observed plenty of fruiting. *Pc* and *Ja* treated plants yielded 3–9 and 2–4 tomatoes per plant, respectively, whereas the control plants did not yield tomatoes. Additionally, the shoot length of OM treated plants were of an average height of ~70 cm for both *Pc* and *Ja* ranging from 40 to 85 cm and 60–90 cm, respectively, while untreated control showed an average of 17 cm with shoot length ranging from 13 to 24 cm.

To elucidate biochemical composition of these OMs, we performed solution-state NMR experiments on the crude extracts to identify various metabolites that attributes to its fertilizing properties. The ^{1}H NMR spectra showed signals in the region 7.34–7.62 ppm which corresponds to aromatic and amines groups of OMs. Another set of signals from 3.21 to 4.62 ppm corresponds to

Table 1
Biometric data of *Solanum lycopersicum* L. (tomato) in response to field trials of OMs.

	Test	Germination rate (%)	Shoot length (mean cm ± SD)	Yield (mean number of fruits ± SD)
1	Untreated control	20 (50*)	16.5 ± 4.3	–
2	Panchagavya (*Pc*)	100 (100*)	67.6 ± 11.4	4.8 ± 1.7
3	Jeevamrut (*Ja*)	70 (100*)	69.8 ± 10.8	3.1 ± 0.9

* Seed germination were observed on 4th week after sowing.

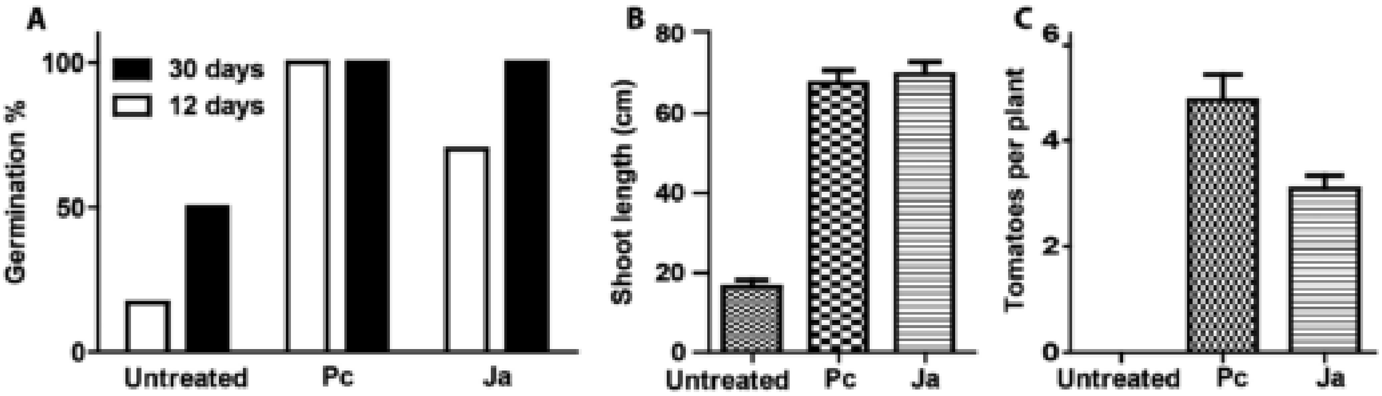

Fig. 1. Bar plots representing field trials of tomato plants with untreated control, 0.3% Panchagavya- *Pc* and 7% Jeevamrut- *Ja*. (A) Germination rates of tomato seeds; 12 days after sowing (DAS) and 30 DAS; (B) Shoot height (cm ± S.D.) of 16.5 ± 4.3, 67.6 ± 11.4 and 69.8 ± 10.3 were observed for untreated, *Pc* and *Ja* tested tomato plants, respectively. (C) Number of tomatoes observed per plant ± S.D) for Pc and Ja were 4.8 ± 1.7 and 3.1 ± 0.9 respectively.

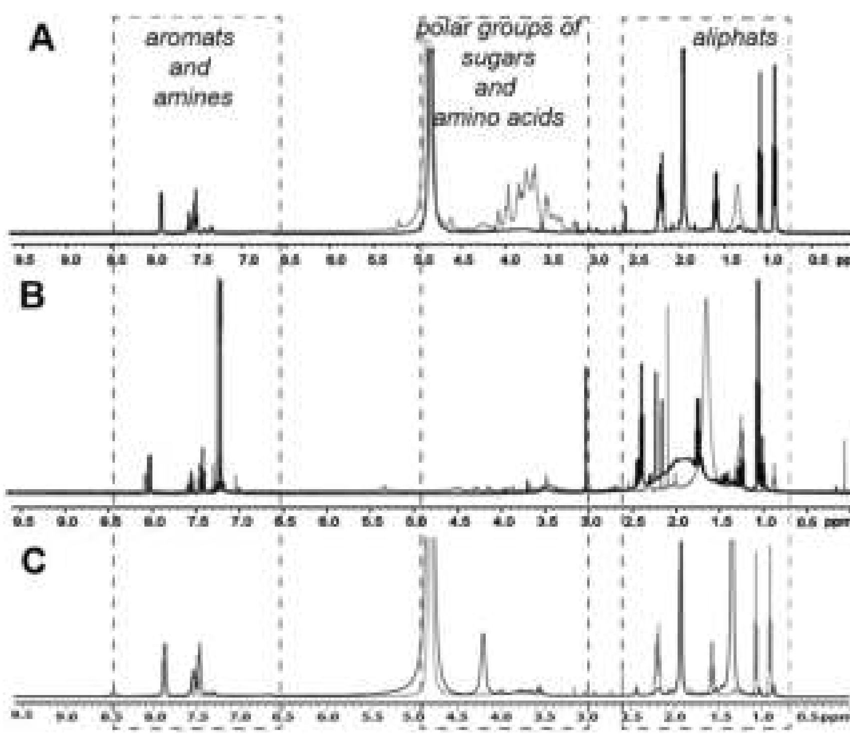

Fig. 2. [1]H NMR spectra of Panchagavya (*Pc*-grey) and Jeevamrut (*Ja*-black) in (A) D_2O and (B) $CDCl_3$. (C) Comparison of [1]H NMR spectra of *Ja* (grey) and Jeevamrut sample from farmers of Rajewadi (*JaR*-black) in D_2O.

polar groups of sugars and amino acids. Up-field 2.5–0.5 ppm, we observed signals suggesting aliphatic constituent of OMs. Fig. 2A represents ^{1}H NMR spectra of OM (*Pc* and *Ja*) in D_2O, which is predominated by several signals in the polar region between 3.21–4.62 ppm, while the spectra of these samples recorded in apolar $CDCl_3$ shows predominance of aromatic and aliphatic groups (Fig. 2B). The two different OM preparations contained a variety of metabolites. The lipophilic phase consisted mainly of hydrophobic metabolic intermediates such as fatty acids (FA), amino acids and several other aromatic compounds (Fig. 2). The hydrophilic phase constituted compounds such as sugars, organic acids and amino acids from carbohydrates and amino acid metabolic pathways. Together, both phases demonstrated that the microbial fermentation of the OMs resulted in a rich repertoire of compounds that are beneficial for plant growth.

To identify the different metabolites and their respective pathways, we fed the list of chemical shift values to the NMR metabolomics database, BMRB. For *Pc*, we identified several amino acids such as glycine, isoleucine, threonine; nucleobase such as guanine; acids from Tri-carboxylic acid (TCA) cycle, which includes acetate, pyruvate and α-keto glutarate; FA such as butyric acid, decanoic acid and other organic acids such as phosphonic acid and phenylacetic acids were present in abundance in the NMR fingerprint. We also identified several key metabolites such as plant growth regulators comprising of indole acetate; indole pyruvate; gibberellic acids; vitamins and co-factors; nucleobases; sugars and their aldehydes with intermediate and low scores that make up many of the biochemical pathways. Similarly, for *Ja*, we observed a similar profile of organic compounds with high scores for acetate, glycine, guanine and other compounds and a lower score for several intermediates and plant growth regulators. Furthermore, except for propionic acid, scores for major FA were a lot lower than *Pc*. Enrichment analysis of the two formulations using Metaboanalyst 3.0 webserver revealed nitrogen metabolic pathways as another major constituent (Fig. 3). Pathways involving sugar and their combinations like amino sugars and nucleotides were

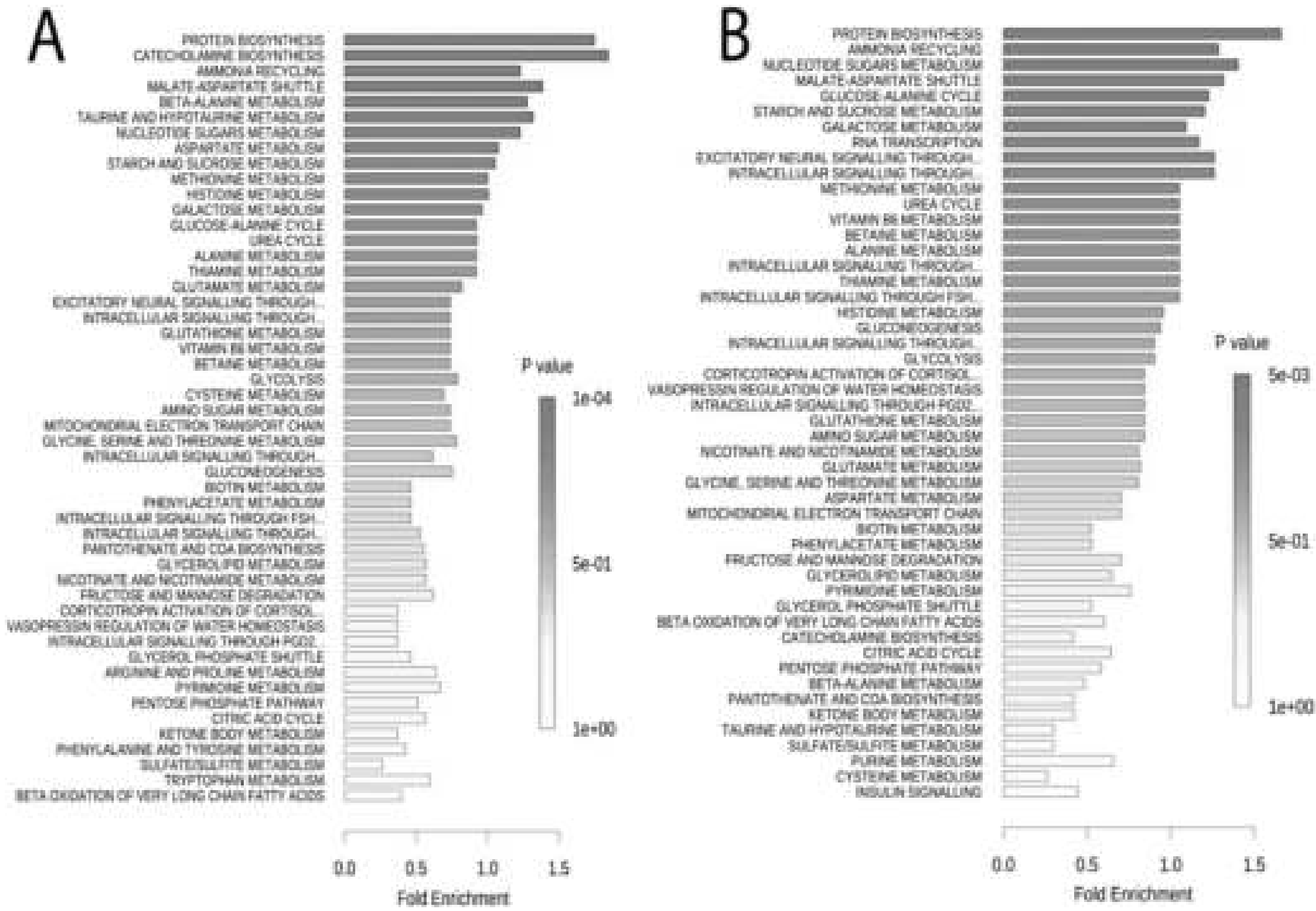

Fig. 3. Enrichment pathways of (A) Panchagavya and (B) Jeevamrut constructed using Metaboanalyst 3.0 from the identified metabolites.

observed at intermediate levels, while specific pathways like tryptophan metabolism which results in production of auxins were observed at the lower end.

Apart from the useful compounds which can act as plant stimulants we also observed in the OM preparations, presence of persistent organic polluters like benzofuran, 4-chlorophenyl acetate (insecticide) and cacodylate (herbicide) with high scores. Other compounds such as atrazine (herbicide) and 2,3-dichloro benzoic acid (fungicide) with medium and low scores respectively, were also identified.

4. Discussion

Organic fertilizers like *Pc* and *Ja* have been previously tested on several crops. Based on the microbial flora that has been (Ali et al., 2011; Gore and Sreenivasan, 2011), reported earlier, suggest that these liquid formulations contain macro- and essential micro nutrients. However, the exact nature of biochemicals in the formulations has remained unstudied. We identified many components that included several plant-growth hormones, nucleobases, vitamins, amino acids, reducing sugars, gluconic acid and other organic acids. To study the beneficial effects of our preparations, we tested the efficiency of these products in field on tomato crops. Firstly, the OMs enhanced seed germination over controls by 2-folds due to the presence of gibberellic acid (Bottini et al., 2004), which was identified by metabolomics study in the OM preparations. Secondly, it promoted rapid development of the shoot and flowers within two months; and tomatoes by 3rd month in comparison with control plants which were short and showed late fruiting. These can be attributed to auxins (Teale et al., 2006); nucleotides that serves as precursors for cytokinins (Schafer et al., 2015), readily utilizable amino acids for nitrogen source, sugars and acids for energy and linking with various metabolic pathways (Kamilova et al., 2006a, 2006b).

Construction of biochemical pathways indicated role of TCA cycle, amino acid and sugar metabolism as the major feed for several metabolites. Earlier reports indicate variation in number and types of microbial loads (Ali et al., 2011; Gore and Sreenivasan, 2011), which is not uncommon as these OMs are prepared from animal wastes and plant materials that are locally available. Hence, they may contain different organic compounds in varying proportions because of bacterial metabolomes and myriad number of signalling pathways. To ascertain our findings, we performed NMR based metabolomic studies on Jeevamrut (*JaR*) sample, from farmers of Rajewadi, Sangli district, Maharashtra, India. We compared the ^{1}H NMR spectral pattern between *Ja* and *JaR*. We observed similar peak profiles but with different intensities (Fig. 2C). This highlights the fact that both *Ja* and *JaR* contain similar metabolites, hence similar biochemical pathways but with varying concentrations. Our results suggested that in the final mixture, TCA cycle acts as major metabolic signalling pathways to feed nitrogen containing metabolites and other organic acids. As sugars are readily metabolized, they are observed in lower amounts in the final preparations. In *Ja*, most FAs were observed at lower scores compared to *Pc* because *Pc* is prepared using cow milk and clarified butter. All these constituents taken together reflect the efficacy of *Ja* and *Pc* OMs.

While these results show a promising story in the use of OMs, we unfortunately noticed traces of herbicides and fungicides, which could have arisen due to contaminated feed for animals. Alternatively, these harmful compounds may have also come from other plant materials that were used in the preparations. For instance, many of these compounds are used in sugarcane farming; since we have used jaggery in all preparation, it may be one of the sources of contaminants. We observed presence of various pesticides, some of which were identified with a high score, while others at intermediary and low score. While comparing our results with those of *JaR*, we observed fewer compounds with lower scores such as Dibenzofuran, 2,4,5-trichlorophenoxyacetic acid (auxin herbicide) intermediate and atrazine. On communication with the farmers of Rajewadi, it came to light that they stopped applying any chemical pesticides on their fields for the last 14 years and have been feeding cattle with plant materials generated in their own farms. These findings explain presence of identified pesticides in *Ja* and *Pc* with a higher score as compared to *JaR*. Regardless, the presence of these compounds serve as a drawback in residue free farming, but can be considered as a preliminary example that repeated use of OMs can eventually lead to their reduction from soil.

5. Conclusion

While the results are encouraging for promoting organic farming, it still highlights one major problem of bioaccumulation and residue free farming. Since pesticides which are difficult to degrade have been extensively used, they get accumulated in our environment and life forms and persist in nature. Clearly our results demonstrated profound success of using OMs that promote plant growth and fruit yield. We still have to devise technology to remove pesticides from our environment and promote better health among individuals and for food export. At the moment, NMR based metabolic profiling can provide as a facile and cost-effective tool in identifying various metabolites and other organic chemicals present in the ecosystem.

Authors contribution

NC and AAC formulated the idea and drafted the manuscript. DUU, RVB and AAC performed the experiments. DUU, SKU and AAC analysed the results.

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