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Study on the microbiological potential of biofertilizer applied on *Brassica oleracea* (cauliflower)

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Plant nutrients are vital component of sustainable agriculture as they are essential for the production of crops and healthy food. Chemical fertilizer or pesticides may have huge influence to increase the heavy metal and resistant microbes in soil as well as in crops. In order to provide an experimental evidence on the positive impact of bio-fertilizer instead of chemical fertilizer on agricultural field, present study attempted to collect 50 samples of *Brassica oleracea* (25 were treated with bio-fertilizer and 25 were chemically treated) from different agricultural land of rural area in Bangladesh. The samples were processed to examine the microbiological and clinical aspects of both bio-fertilizer and chemical fertilizer on vegetables through several common, traditional and replicable cultural and biochemical tests. Both samples were found to be contaminated with total viable bacteria and fungi up to the range 10^8 & 10^6cfu/g, respectively. The elevated range of pathogenic contamination (*Staphylococcus* spp., *Bacillus* spp., *Pseudomonas* spp.) was found in both samples within the range of 10^2 to 10^6cfu/g. In case of biofertilizer treated vegetable the contamination of *Staphylococcus* spp. was prominent up to 10^6 cfu/g and the same existence was found for chemically treated vegetable. *Bacillus* spp. and *Pseudomonas* spp. were found 10^4 & 10^5 cfu/g, respectively in biofertilizer treated vegetable while the contamination was noticed up to 10^2 & 10^4 cfu/g in chemically treated vegetable respectively. Another important era of this study is drug resistant pattern, most of the isolates exhibited resistance against commonly used antibiotics while several isolates were noted to be multi-drug resistant (MDR). The drug resistance strains were remarkably high in chemically treated vegetable whereas maximum antibiotics were extremely effective against the bacteria isolated from biofertlizer treated vegetable.

Key words: Biofertlizer; Chemical fertilizer; Vegetable; MDR; Public health

Improper disposal of wastes such as land disposal of municipal and industrial wastes, automobile emissions, mining activity, as well as applications of fertilizers and pesticides are great concerns in today's world (1-4). Composting is considered as the only eco-friendly and natural process of recycling or microbial decomposition method of organic matter under controlled conditions which can convert this huge amount of wastes into a valuable resource (5-7). Since composting is a microbiological process, different types of mesophilic, thermotolerant and thermophilic aerobic microorganisms (e.g. bacteria, actinomycetes, yeasts, and fungi) are involved in the composting process (6-8). Comparing to chemical fertilizer or mineral fertilizer, organic manure or compost is an inexpensive biofertilizer that increases soil organic matter, the water-holding capacity of coarse-textured sandy soils, improves drainage in fine-textured clay soils, provides a source of slow release nutrients, reduces water erosion, and promotes growth of earthworms and other beneficial soil organisms (9).

However, previous studies have reported that several food-born diseases were associated with the consumption of raw fruits & vegetables contaminated by manure (10-13). The major reason behind the contaminated vegetables and fruits as well as accumulation of heavy metals in the soils and vegetables is the long-term use of excessive chemical fertilizers and organic manures in the bare vegetable field and the greenhouse vegetable field (14). High fertilizer applications causes acid atmospheric deposition and decrease in pH and thus increases heavy metal availability, aggravating the problem of deteriorating food quality, metal leaching, and impacts on soil organisms (15). So, the agricultural or domestic use of this manure can increase the risk of disease transmission by direct contact of humans with the material or by contamination of food crops or by adding to environmental contamination which may maintain diseases in the food of animal population (16). Moreover, transmission of drug resistance virulent genes of these soil microflora can act as carrier in the transmission of disease to human through the environmental factors such as water, food residue and waste material (17-20). Therefore, our current investigation attempted to ponder the microbiological aspect of biofertilizer and chemical fertilizer on raw *Brassica oleracea* along with the drug resistance pattern of the isolates from vegetable.

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MATERIALS AND METHODS

Study area, sampling and sample processing. Total 50 samples of *Brassica oleracea* (25 were grown with bio-fertilizer and 25 were grown using chemical fertilizer) were randomly collected from different rural area in Bangladesh during January 2014-March 2014 following standard protocol (21).

Estimation of total viable bacteria and fungi. The enumeration was performed by using 0.1 ml of each sample from the dilution 10^{-3} was spread onto nutrient agar (NA) and Sabouraud Dextrose Agar (SDA) for total viable bacteria (TVB) and total fungal load, respectively. After that the nutrient agar (NA) and Sabouraud Dextrose Agar (SDA) plates were incubated at 37 ^{o}C for 24 hours and at 25 ^{o}C for 48 hours for the detection of total viable bacteria (TVB) and total fungus respectively according to the standard guideline (22).

Estimation of coliform count and fecal coliform count (FCC). An aliquot of 0.1 ml of each sample was spread on to MacConkey agar, and membrane fecal coliform agar plates for the estimation of coliform (*E. coli* and *Klebsiella* spp.) and fecal coliform (FCC) respectively. For coliform count, all plates were incubated at 37 °C for 24 hours while for estimating the fecal coliforms, incubation was carried out at 44.5 °C for 24 hours. Eosin methylene blue agar media was further used for the observation of production of green metallic sheen (if any) to ensure the specific characteristic of *E. coli* strains (17,18).

Estimation of *Staphylococcus* spp., *Pseudomonas* spp. *Clostridium* spp. *Bacillus* spp. and *Listeria* spp. Same amount of samples as described above was spread on to mannitol salt agar, cetrimide agar Starch agar and *Listeria* media for the isolation of *S. aureus*, *Pseudomonas* spp., *Bacillus* spp and *Listeria* spp., respectively. Afterwards, plates were incubated at 37 °C for 24 hours. For the isolation of *clostridium* 1 ml of each blended sample were mixed in sterile normal saline in a ratio of 1:8 followed by heating at 80 °C for 15 minutes in order to kill vegetative cells of the microorganisms (17,18). Furthermore, 1ml of each samples were introduced into 9 ml of fluid thioglycolate broth and incubated for 4 hours at 37 °C. Then 0.1 ml of each sample from this enriched broth was poured on *Clostridium* isolation agar plates according to pour plate method and were incubated at 37 °C in anaerobic condition for 48 hours (23).

Estimation of *Salmonella* spp., *Shigella* spp. *and Vibrio* spp. For the isolation of VBNC Salmonella spp., Shigella spp., and Vibrio spp. 1ml of each samples was inoculated into Alkaline peptone water (APW) and Selenite cystain Broth (SCB) for enrichment and incubated at 37°C up to 6 hours. Afterward, 0.1 ml of each sample from the broth was introduced on selective media such as Salmonella, Shigella agar and the Thiosulfate Citrate Bile Salt Sucrose agar media respectively and plates were incubated at 37 °C for 24 hours (24).

Confirmatory Biochemical tests. For the final identification of all isolates, several biochemical tests were performed including the triple sugar iron test, motility indole urease test, methyl red test, Voges Proskauer test, indole utilization test and the oxidase test (table 2) (22).

Determination of antimicrobial susceptibility of the isolates. The pathogenic isolates were examined for antibiotic susceptibility traits (either drug resistant or sensitive) by disc diffusion assay on Mueller-Hinton agar (Difco, Detroit, MI) against commonly used antibiotics following the standard protocol (23,25,26). Lawns of bacterial suspensions including *Escherichia coli*, *Pseudomonas* spp., *Vibrio* spp., *Staphylococcus* spp.and *Salmonella* spp. (turbidity compared with the McFarland standard OD_{600}-0.5) were prepared and introduced on to Muller Hinton agar. Antibiotics used in the study included polymixin B (300 unit), Kanamycine (30 µg), methicillin (30 µg), streptomycin (10 µg), vancomycine (30 µg), gentamycine (10 µg), nalidixic acid (30 µg), azythromycine (15 µg), penicillin G (10 µg), erythromycin (15 µg), amoxicillin (30 µg), ceftriaxon (30 µg), ciprofloxacin (5 µg), ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg) and cefixime (5 µg). All plates were incubated at 37 °C for 12-18 hours and examined for formation of the zone of inhibitions (mm) (27).

RESULTS AND DISCUSSIONS

In developing countries like Bangladesh agriculture sector plays an important role for developing the socio-economic status of the farmer as well as the country (28). To ensure the better production of crops, reduce the health and environmental risk and soil degradation scientists suggested that the use of agriculture fertilizer (bio-fertilizer) is more effective than the chemical fertilizer (29). Present study showed that the both samples were found to be highly contaminated with total viable bacteria and fungi up to the range 10^8 & 10^6cfu/g respectively (Table 1).

TABLE 1. Comparative microbial load (cfu/g) within the biofertilizer treated vegetables and chemical fertilizer treated vegetable

Microorganism	Biofertilizer treated (*Brassica oleracea*) n=25	Chemical treated (*Brassica oleracea*) n=25
TVBC	6.3×10^7	2.6×10^8
Fungi	6.6×10^6	1.3×10^5
Coliform	0	0
Fecal coliform	0	0
Staphylococcus spp.	1.0×10^6	1.5×10^6
Bacillus spp.	3.3×10^4	2.7×10^2
Pseudomonas spp.	1.4×10^4	2.1×10^3
Vibrio spp.	0	0
Salmonella spp.	0	0
Shigella spp.	0	0
Listeria spp.	0	0
Clostridium spp.	0	0

TVBC = Total viable bacterial count
The average counts have been shown and the results were reproducible

Although the samples were free from coliform, fecal coliform bacteria but existences of pathogenic flora such as *Staphylococcus* spp., *Bacillus* spp., *Pseudomonas* spp., were also found in all the samples of both biofertilizer treated and chemically treated (Table 1). *Vibrio* spp., *Salmonella* spp., *Shigella* spp., *Listeria* spp. and *Clostridium* spp. were not cultivated from the both samples.

In case of biofertilizer treated vegetable the contamination of *Staphylococcus* spp. was prominent up to 10^6 cfu/g and the same existence was found for chemically treated vegetable. *Bacillus* spp. and *Pseudomonas* spp. were found 10^4 & 10^5 cfu/g respectively in biofertilizer treated vegetable while the contamination was noticed up to 10^2 & 10^4 cfu/g in chemically treated vegetable respectively (Table 1). All the isolates found in the tested samples were biochemically confirmed through several test. As described in previous studies the excessive implementation of chemical fertilizer and organic manure are directly responsible to reduce the soil quality, nutritive value of the crops and shelf life (30). However, several researchers have demonstrated that the combined use of chemical and biofertilizer has beneficial impact on the mitigation of secondary and micronutrients in fields and also these heavy metals or biomass may remain in vegetables and fruits (31-35).

Frequency of antibiotic resistant isolates. The drug resistant profile of the isolates from both cases was introduced against 17 antibiotics. All the isolates found in this study showed resistance against at least two antibiotics but the resistance properties of the isolated bacteria were very high in chemically treated vegetable respectively (Table 2). All the isolates found in the tested samples were biochemically high in chemically treated vegetable (Table 2). In case of biofertilizer treated sample, *Staphylococcus* spp. and *Pseudomonas* spp. were found to be 100% resistant against Penicillin G, Amoxicillin, Ampicillin, Tetracycline and Methicillin, Penicillin G, Ampicillin respectively while *Bacillus* spp. shower 100% resistance against Polymixin B, Penicillin G and Amoxicillin. Maximum antibiotics were highly effective against the bacteria isolated from Biofertilizer treated vegetable (Table 1). Conversely, all the isolates were found to be resistance against almost all antibiotics in case of chemically treated vegetables (Table 2). *Staphylococcus* spp. was found sensitive only against Methicillin, Streptomycin Gentamycin & Azythromycin and *Bacillus* spp. was sensitive against Streptomycin, Gentamycin and Azythromycin. *Pseudomonas* spp. was found to be sensitive only against Streptomycine and Gentamycin (Table 2). All 3 pathogen isolated from chemically treated vegetable showed their resistance against multiple drugs which indicated that the strains were multi drug resistant (MDR). Above describe findings clearly demonstrated that the implementation of chemical fertilizer on vegetable significantly increases the bacterial resistance rather than the biofertilize. Consumption of these drug resistant bacteria through different vegetable and fruits may lead serious food born illness in human and also reduce the natural immunity of the individual.

The resistance might be due to genetic, mechanistic or epidemiologic considerations, cross resistance prominence and metabolic changes (36-38). According to the microbial genetic study, mutation is one of the major reasons which can accelerate the bacterial resistance by altering the genetic code in DNA due to the influence of several chemical agents and transferring the resistant gene to sensitive one from a virulent pathogen via conjugation (38).

Finally, in addition to the microbiological prospective of biofertilizer and chemical fertilizer, it's necessary

TABLE 2. Antibiotic resistance/sensitivity pattern of the isolates

Antibiotic	Disc content	Isolates											
		Biofertilizer treated						Chemically treated					
		Staphylococcus spp. *n=50*		*Bacillus* spp. spp. *n=50*		*Pseudomonas* spp. *n=50*		*Staphylococcus* *n=50*		*Bacillus* spp. *n=50*		*Pseudomonas* spp. *n=50*	
		R (%)	**S (%)**	**R (%)**	**S (%)**	**R (%)**	**S (%)**	**R (%)**	**S (%)**	**R (%)**	**S (%)**	**R (%)**	**S (%)**
Polymixin B	300 units	0	100	100	0	0	100	100	0	100	0	100	0
Kanamycine	30 µg	0	100	0	100	0	100	100	0	100	0	100	0
Methicillin	30 µg	0	100	0	100	100	0	0	100	100	0	100	0
Streptomycine	10 µg	0	100	0	0	0	100	0	100	0	100	0	100
Vancomycine	30 µg	0	100	0	100	0	100	100	0	100	0	100	0
Gentamycine	10 µg	0	100	0	100	0	100	0	100	0	100	0	100
Nalidixic acid	30 µg	0	100	0	100	0	100	100	0	100	0	100	0
Azythromycine	15 µg	0	100	0	100	0	100	0	100	100	0	100	0
Penicillin G	10 µg	100	0	100	100	100	100	100	0	100	0	100	0
Erythromycine	15µg	0	100	0	100	0	100	100	0	0	100	100	0
Amoxicillin	30µg	100	0	100	100	0	100	100	0	100	0	100	0
Ceftriaxone	30µg	0	100	0	100	0	100	100	0	100	0	100	0
Ciprofloxacine	5µg	0	100	0	0	0	100	100	0	100	0	100	0
Ampicillin	10µg	100	0	0	100	100	0	100	0	100	0	100	0
Tetracycline	30µg	100	0	0	100	0	100	100	0	100	0	100	0
Chloramphenicol	30µg	0	100	0	100	0	100	100	0	100	0	100	0
Cefixime	5µg	0	100	0	100	0	100	100	0	100	0	100	0

R = Resistant
S = Sensitive
All the experiments have been done three times and the results were reproducible. Average data have been shown.

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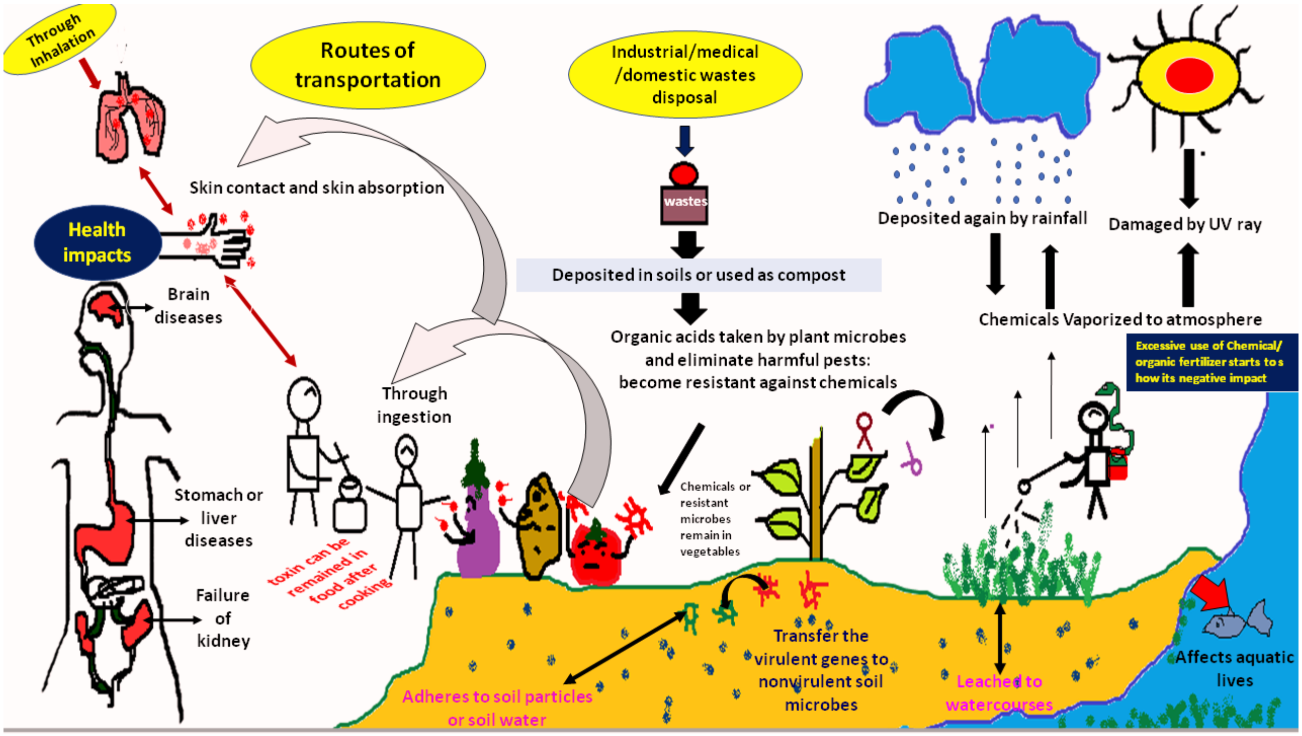

FIG. 1. Effects of chemical fertilizer on agriculture field as well as environment. Excessive use of chemical fertilizers in the agricultural fields may pose a negative influence on environment; some amounts of these fertilizers become vaporized in the open atmosphere in the presence of sunlight or UV ray. These vaporized chemicals can be deposit again in the soil by rainfall or acid rain. Some are leached into the soil and watercourses and affects aquatic lives of nearest river.

to provide a complete schema on the bad impact of chemical fertilizer towards the soil as well as environment. This study attempted to demonstrate a simple overview by which researcher/expert can explain the whole things to the general people as well as the farmers very effortlessly (Figure 1).

Excessive use of chemical fertilizers in the agricultural fields may pose a negative influence on environment; some amounts of these fertilizers become vaporized in the open atmosphere in the presence of sunlight or UV ray. These vaporized chemicals can be deposit again in the soil by rainfall or acid rain. Some are leached into the soil and watercourses and affects aquatic lives of nearest river. In the presence of these chemicals for long periods in the soil, soil bacteria can become virulent by developing resistant genes against these chemicals. Similar results can found in case of deposition of wastes in the soil. On the contrary, plant microbes uptake the chemicals or organic acids from the fertilizer and become virulent (Figure 1). When human ingest these vegetables, the virulent microbes are also introduced in their body through ingestion, skin contact (handling) or inhalation. This can cause severe health impacts like blood disorder, brain disease or failure of kidney.

CONCLUSION

Increased crop production and soil fertility largely relies on the type of fertilizers used in the land as supplement nutrients for plants. Biofertilizers differ from chemical and organic fertilizers for its availability, simple production procedure and low cost rate. Regardless of methods, microbes present in biofertilizer will decline and be eliminated in a very short time after application in the field. At present, overuse or misuse of chemical fertilizers in agriculture is one of the major motive of environmental deterioration as well as contaminated food products. Overall, the present study revealed that biocompost or biofertilizer imparted no added effect in context of microbiological quality on the vegetables on which they were applied. In this point of view, it can be concluded that biocompost can easily be applied to the field instead of chemical fertilizer considering its economic benefit together with environmental sustainability as might be pretense by the chemical fertilizers. Finally, current investigation revealed that the increasing state of drug resistant bacteria due to the improper use of chemicals in agricultural land might responsible for serious obstacle in proper medication of the diseases which could potentially become a public health threat. Efficient plant nutrition management should ensure both enhanced and sustainable agricultural production and safeguard the environment. Chemical, organic or microbial fertilizer has its advantages and disadvantages in terms of nutrient supply, soil quality and crop growth. Developing a suitable nutrient management system that integrate the use of these kinds of fertilizers may be a challenge to reach the goal of sustainable agriculture; however much research is still needed especially for the agricultural based country to ensure the agriculture linked health safety.

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