Reviewer comments:

Line 70. physio-chemical. Typo

Line 74-85. The terms in your experimental design was rather confusing. When you use an experimental design, you need to use common terms appropriately.

a. The fundamental question needs to be explained is why did you use RBD. What made different among blocks? You have to mention some reasons
b. You have eight "Plots". Right? Is it different from the term "Block"? how many blocks did you have?
c. You have eight treatments, Right? Normally RBD includes a factor consisting of levels (that is what you called eight treatments).
d. You said you had six replicates? In RBD, these are not true replicates. In your design replicates = number of blocks.
e. What is the size of your experimental unit? You mentioned six replicates for each treatment. I think this is the size of experimental unit in formal experimental design. Exp unit = 6 plants.
f. Please clarify

Application of egg shell with fortified vermicompost in *Capsicum* cultivation: A strategy in waste management

Abstract

Purpose Chicken eggshell ES) is a global biowaste product of poultry industry and house hold things, however, it is an enriched source of calcium required for plant biomass enhancement. Therefore, the present study has been carried out to asses the potentiality of the combination of chicken eggshell with vermicompost (VC) and chicken feather protein hydrolysate (CFPH) on growth and yield improvement of *Capsicum* plants.

Methods A field study was conducted through randomized block design (RBD) with eight treatments having six replicates for each. Principle Component Analysis (PCA) have performed to analyze the yield related parameters of plant. Nutritional components of VC and ES were also analyzed.

Results The PCA analysis of the field experiment has showed that the combination of ES, CFPH and VC (in a ratio of 100:10:3) remarkably increases the agronomic parameters of capsicum plant about four folds as compared to its chemical counterpart and control, while together VC and ES strongly influences the characteristics of fruits. Therefore, the execution of amalgam of ES, CFPH with VC could enhance the yield parameters of crops with concomitant reduction in the use of chemical fertilizers.

Conclusion A combination of ES, CFPH and VC could enrich the soil with both micro and macronutrients, and it could also serve as another source of organic compost for plant growth-promotion.

Keywords Egg shell, calcium, vermicompost, chicken feather protein hydrolysate, *Capsicum*

Introduction

Agriculture with the aid of organic fertilizer is an environment safety measure. It provides an exceptional alternative for waste management and their recycling (Biswas et al. 2021). The application of organic fertilizer provides plants with valuable nutritional sources and improves the physical and chemical status of soil and ultimately increases crop yield (Andhare et al. 2019). Vermicompost (VC) is the organic manure widely used for crop production globally with high market demand. It has improved soil health, plant productivity and disease resistance potential. There are several reports in support of the view that application of VC as soil supplement could increase the productivity of several crops and plants (Rekha et al. 2018; Bijeta et al., 2018; Hector Ardisana et al. 2020; Biswas et al. 2021). Chicken feathers are the waste generated from poultry industry due to large consumption of chicken worldwide. The chief component of feather is protein keratin which is tough, insoluble in nature. The recalcitrant nature of feathers leads to wastage of feather protein. However, these feathers could be hydrolyzed through biochemical, thermal and microbial enzymatic approach to produce value added chicken feather protein hydrolysate (CFPH) which could serve as source of nitrogen fertilizer for plant growth and development (Genç and Atici, 2019; Biswas et al. 2021). Egg shells (ES) are another global poultry biowaste products generated from hatcheries, household and

convenience-food centers. Land fill disposal problem of huge quantity of egg shells leads to environmental pollution due to its foul odour that encourages flies and corrosiveness (Gaonkar and Chakraborty, 2016; Nagamalli et al. 2017; Andhare et al. 2019; Thakur et al. 2019). About 10-11% of the total weight of the egg is occupied by eggshell which contains about 96% of calcium carbonate and a number of trace elements. However, the utilization of eggshell waste as biofertilizer source for cultivation is another scientific practice. Eggshell can be used for several other purposes like dental therapy, as biodiesel. It is rich in both organic and inorganic materials including macro and micronutrients such as potassium, nitrogen, calcium, magnesium, phosphorus, chloride and zinc which are essential for plant growth promotion. Crushed eggshell fertilizer provides the soil with calcium along with other nutrients contained in them. The major advantage of using crushed eggshell is that its preparation requires less energy however, the disadvantage lies in the time required for their degradation before providing nutrients for plant growth (Wijaya and Teo, 2019). A study observed that red clover plants when grown with eggshell fertilizer grew 10 mm larger than the plants without it (Wazir et al. 2018). Pepper (*Capsicum annuum* L.) is the second most favored horticultural plant and is grown over 40 countries in the world. It is preferred for its flavour, high nutritional value, and is rich in fiber, antioxidants and vitamins. Pepper is recommended for its therapeutic potential in the treatment of inflammations, gastric ulcers and digestive disorders (Hector Ardisana et al. 2020).

In the present study, enriched vermicompost supplemented with chicken eggshells and chicken feather protein hydrolysate have been used as organic fertilizer to evaluate their influence on agronomic parameters of capsicum plant, leaf chlorophyll content and fruit quality management.

Materials and methods

Collection of chicken egg shell and preparation of egg shell dust

The chicken egg shells were collected from the fast-food center and house hold sources of Raiganj town, Uttar Dinajpur district, West Bengal, India. They were cleaned with the help of normal tap water repeatedly to remove any kind of impurities and then with warm water to reduce microbial contaminants and after that dried for a couple of days under sunlight at 30-35℃. They were grinded to a fine powder with a mixer grinder and this powder was further kept at room temperature for their use in experiment.

Production of VC and CFPH

VC and CFPH were prepared according to the methods as depicted by Biswas et al. (2021).

Study of physicochemical parameters of VC and egg shell dust

The nutritional parameters such as Carbon, nitrogen, phosphorus, potassium, hydrogen, calcium, magnesium, copper, iron, zinc, manganese, lignin and other heavy metals such as cadmium, chromium, lead of VC and egg shell dust were analyzed through while organic components such as cellulose, hemicellulose, crude fiber of VC were analysed through Atomic Absorption Spectrometer, Thermo Fisher Scientific Inc. United States (Nurdiawati et al. 2019).

Test plant, site, experimental layout and treatments

The field experiment was conducted at Professor A. K. Bothra Environment Conservation Centre (PAKBECC), Raiganj University, Raiganj - 733 134, Uttar Dinajpur, West Bengal, India (25.6329° N, 88.1319° E) from November 2020 to March 2021. The physio-chemical properties of the soil were measured earlier (Biswas et al. 2021). Capsicum plants (*Capsicum annuum* L.) were selected for the field experiment and eight plots were prepared. Initial germination of Capsicum var. Dalisha 2037 (F1 hybrid) seeds were carried out in earthen pots for about 20 days till the emergence of two opposite leaf initials and after that they were transferred to the field for further growth and development. The experimental field was arranged in a randomized block design (RBD) with eight treatments. All plants were separated from the other by a distance of 25 cm. There were six replicates for each treatment within each block. The blocks were located 1.0 meter apart from each other and there were six plants per block in each plot. The eight treatments were as follows:

(T_0) Control,

(T_1) VC; 5 t per ha

(T_2) ES; 5 gm per plant

(T_3) CFPH; 2 ml/ L

(T_4) 1000 gm VC + 30 gm ES per block

(T_5) 1000 gm VC + 100 ml CFPH per block

(T_6) 1000 gm VC + 100 ml CFPH + 30 gm ES per block

(T_7) RDF- 200:250:250 kg of NPK per ha

Prior to application, VC, CFPH and ES were disseminated in water at the rate of 1:1:1. The plots were watered every day to maintain required amount of moisture in the soil.

Morphological analysis of capsicum plant

The experimental data on agronomic parameters, *i.e*., average plant height (in cm), number of leaves per plant, length and breadth of leaves, leaf area (square cm), fresh and dry weight of leaf (g), flowers and fruits number, fresh weight and crop yield were determined at the regular time interval of 20 days up to the harvest. All data of the field experiment were analyzed through R software (Team 2013).

The leaf area was calculated by the following equation-

Leaf area (cm2) = K× length× breadth

Where = Kemp's constant (dicot leaves = 0.66) (Radha and Karthikeyan, 2019).

For capsicum, additional agronomic parameters including average number of fruits per plant, average fruit length (cm) and breadth (cm), average fruit weight (g) and yield per plant (kg/plant) were measured. The total chlorophyll present in the experimental plant leaf were estimated by Acetone method.

Biochemical analysis of fruits

Extraction and estimation of protein was done by Biuret method and carbohydrate estimation was performed according to DNS method. Besides pH, total titrable acidity, tannin content (mg/g) of fruits were also analysed (Das et al. 2014).

Statistical analysis

Principal component analysis (PCA) biplot analysis was carried out using R software (Team, 2013) for visualizing the dataset consisting of observations and analyzing the impact of different treatments on plant growth. The built-in function 'PCA' available in FactoMineR package (Lê et al. 2008) was used for computing PCA. As the mean and standard deviation of the variables were largely different, scaling of the dataset was carried prior to analysis which utilized the R base function 'scale' thereby making the variables comparable. The biplot was constructed using the function 'fviz_pca_biplot', provided in the FactoMineR package.

Results and discussion

Physicochemical parameters of VC and egg shell dust

Physico- chemical properties of the egg shell dust (Fig. 1A-B) and vermicompost (that was produced at the PAKBECC (VERMITECH), Raiganj University, Raiganj) has represented in Table 1. The parameters studied includes organic carbon, nitrogen, phosphorus, potassium, hydrogen, calcium, magnesium, copper, iron, zinc, manganese, organic components such as cellulose, hemicellulose, crude fiber, lignin and other heavy metals such as cadmium, chromium, lead. Results have shown that vermicompost produced from organic waste has shown a comparatively higher amount of organic and inorganic nutrients compared to the eggshell dust (Table 1).

Field application results of different treatment on growth promotion of capsicum plant

The inertia of the first dimensions indicates whether there are strong correlations between variables and suggests how many dimensions should be investigated (Fig. 3). The first two dimensions of PCA analysis express 88.39 percent of the overall dataset inertia; that is, the plane explains 88.39 percent of the total variability of the individuals (or factors). Because this percentage is so large, the first plane accounts for a significant portion of the data variability. This result is significantly higher than the reference value of 36.32 percent, indicating that the variability described by this plane is quite substantial (the reference value is the 0.95-quantile of the inertia percentages distribution obtained by simulating 2905 data tables of equivalent size on the basis of a normal distribution). According to an estimate of the appropriate number of axes to interpret, the analysis should be limited to the description of the first one. These axes have a higher level of inertia than the 0.95-quantile of random distributions (81.57 percent against 20.24 percent). This finding implies that only this axis carries genuine

information. As a result, the description will be applicable to these axes. PC1 Explained the following variables LN (0.950), LA (0.940), PH (0.940), LB (0.918), LL (0.899), FW (0.874), DW (0.872), FlN (0.871) and FrN (0.855). PC2 described the following variables FW (0.476), DW (0.463) and FrN (0.446).

From the PCA biplot representation (Fig. 3), it was visualized that the bottom right quadrant had treatments T_4, T_5 and T_6, followed by T_7 in the bottom left quadrant while T_1 in the top left quadrant and T_2, T_3 and T_5 in the top right quadrant. From this analysis, the order of the effectiveness of the treatments on agronomic parameters of the plants were as follows- $T_6>T_4>T_5>T_7>T_3>T_2>T_1>C$. The analysis confirms the parameters represented by PC2 *viz.* PH, LN, LL, LB, LA, FrN were strongly influenced by the treatments T_4, T_5 and T_6 while FW, DW and FlN were strongly regulated by treatments T_2, T_3 and T_5.

The PCA dataset consisted of 16 individuals and 12 variables. No outlier was detected in the graph (Fig. 4). The inertia of the first dimensions indicates whether or not there are strong correlations between variables and specifies how many dimensions should be investigated. It would be better to understand the dimensions bigger than or equal to the third one based on these observations. The first two dimensions of analysis express 66.91 percent of the overall dataset inertia; that is, the plane explains 66.91 percent of the total variability of the persons (or variables) cloud. Because this percentage is so high, the first plane accurately portrays the data variability. This result is significantly higher than the reference value of 46.76 percent, indicating that the variability indicated by this plane is quite substantial (the reference value is the 0.95-quantile of the inertia percentages distribution obtained by simulating 2974 data tables of equivalent size on the basis of a normal distribution). The following parameters were explained by PC1 with their respective correlation coefficients provided in parenthesis FW (0.947), FL (0.934), FY (0.919), FB (0.902), Pro (0.891), FN (0.795) with all parameters having p value of <0.001. The second PCA dimension explained the following variables, Tan (0.888), fChl (0.851), Car (-0.630) and iChl (-0.655). However, the variables pH (0.888) and TTA (-0.741) couldn't be explained by PC1 and PC2, rather was explained by PC3. From the biplot representation (Fig. 4), it was observed that the bottom right quadrant had treatments T_6 and T_5, followed by T_1, T_2 and T_7 in bottom left quadrant, while T_3 and control in top left quadrant followed by T_4 in the top right quadrant. From this analysis it can be concluded that the parameters represented by PC2 viz. FY, FB, FL, FW and Pro were exclusively influenced by treatment T_4 while FN, Car, and iChl were regulated by treatments T_5 and T_6 and likewise Tan and fChl were strongly influenced by T_3 as compared to control. The impact of experimental treatments on capsicum fruit as compared to control has been presented in Fig. 2.

The application of VC and CFPH as plant growth promoting agent have been studied earlier. Biswas et al. (2021) observed the potential influence of the combination of CFPH with VC on growth and yield of tomato plants as compared with the recommended dose of chemical fertilizer. The PCA analysis of the field experiment observed that a combination of CFPH and VC proved more efficient in terms of enhancing the germination (84.13 %) and growth parameters in tomato plants. On the other hand, several experiments were conducted to evaluate the effect of eggshells and different organic fertilizers in plant cultivation. An experiment was done to evaluate the influence of eggshells on vegetative growth of cayenne pepper (*Capsicum frutescens* L.). Eggshell organic fertilizer was applied

with a dose of 0 (P0); 45 (P1); 60 (P2); 75 (P3); 90 (P4); and 105 g(P5). Results have showed that P4 treatment performed best for root length and plant wet weight (20.10 cm and 17.96 g, respectively) while P5 treatment gave highest plant height and plant dry weight (54.80 cm and 3.00 g respectively) (Anugrah and Safahi, 2021). Ertürk (2020) used Chlorella Vulgaris algal biomass and eggshell waste (ESW) individually and combination of both the organic source as mineral and nutritional supplement for cultivation of tomato plant in the controlled atmosphere of the laboratory. The results have showed that combined treatment of ESW + algae had increased the calcium content in tomatoes as compared to other treatments. Another study using organic fertilizer was conducted to observe the effect of a bovine manure vermicompost leachate (six dilutions) on growth, and yield of pepper (*Capsicum annuum* L.) hybrid Nathalie. The experiment was conducted in randomized blocks with four replications. The results showed no significant differences in the growth parameters of pepper among the experimental treatments. Moreover, the estimated yield from all the treatments of BMVL was quite similar with each other. This experiment introduces bovine manure vermicompost leachate as alternative organic fertilizer for pepper cultivation with a simultaneous reduction in environmental contamination (Hector Ardisana et al. 2020). Wijaya and Teo (2019) observed the effect of eggshell in its liquid form in influencing the plant growth parameters in case of sweet basil compared to the commercial one. The results of this study proved this liquid organic fertilizer to be compatible with its chemical counterpart in terms of increasing the plant height and this is might be due to the nutrients such as nitrogen, potassium, chloride in eggshell tea fertilizer. Moreover, it has also been found that eggshell fertilizer is more productive in its liquid form than its solid form. Andhare et al. (2019) studied the use of egg shell in combination with banana peels (*Musa sapientum*) and duckweed (*Lamna minor*) on wheat crop in pot culture and it was found that wheat crop grows very rapidly when supplemented with the organic fertilizer and there was an increment in agronomic parameters as compared to control without organic fertilizer. Besides a remarkable difference in soil pH was also observed (from 5.7 to 6.9). It was concluded that the positive results were due to the presence of increased concentration of essential nutrients needed for plant growth and development. Wazir et al. (2018) observed the influence of various house hold wastes as organic fertilizers on growth and yield of potatoes and peas. The overall yield improvement of both the plants increased and the average plant height (14.75 cm) was found to be greater in plants treated with egg shell powder. Treatment with egg shell powder was also found to increases the number of leaves, nodes, branches, the average number of large size tubers (2.25) per plant and leaf area. These results concluded that among these organic fertilizers, eggshell powder, banana peel and tea waste were proved to be the best for the cultivation of potato and pea. A similar experiment was conducted on red chili to evaluate the effect of rice husk ash and ES on the growth and yield of plants however, results have showed no significant interaction between the two treatments in increasing the number of productive branches, number of fruit / plant and weight of fruit/ plant. Moreover, it was also reported that rice husk ash gave the observable effect on increasing the plant height, plant growth and the number of leaves and the effective dose was 50 g/ plant. While the ES gives real effect on yield of red pepper at the effective dosage of 30 g/ plant (Kurniastuti 2018). Another experiment was performed during 2015 - 2016 to evaluate the effect of growing media on yield and growth of capsicum cv. Orobelle. Results have showed that the medium composition (Soil + Cocopeat + VC + FYM) in a ratio of 2:1:0.5:0.5 proved to be admirable over other growing media statistically for maximum growth of plants. It also recorded the maximum fruit

length (10.25 cm), average fruit weight (192.91 g), fruit yield (2.81 kg/plant). Therefore, it was inferred that application of cocopeat, vermicompost, and FYM improved soil properties and enhanced nutrient supply and accumulation of more metabolites to plants (Bijeta et al., 2018). Zahan et al. (2018) carried out a study for 109 days from 6th November, 2016 to 23rd February, 2017 to assess the response of tomato plants to calcium supplement through egg shell in aquaponics. For each replication two tomato saplings were planted. The chicken egg shell powder was added in treatment T1 (1kg/decimal) and T2 (1.5kg/decimal) while T3 was control. The higher production of tomato (73.58 tons/ha/109 days) was observed in T1 which signifies the potential of egg shell powder as source of calcium. This treatment also gave the highest mean weight of the plant (164± 46.16 g), highest root height and the weight (36.33±5.68 cm and 54.5±10.13 g, respectively). It was also reported that supplementation of saline soil with vermicompost and ES could reduce soil salinity (about 77%) and helps in germination and growth of plants as observed in lettuce plant (Zurbano., 2018). A similar experiment was conducted on yield of okra and soil fertility with the application of biomixture including spent coffee ground and milled egg-shells against inorganic fertilizer as control. Results have showed that treatments provided with 10% and 5% of the biomixture gave highest yield of Okra and was 16707 and 16102 g/plant/pot respectively with simultaneous increase in organic matter with concomitant bacterial and fungal cell counts in soils as egg shells contain a large amount of nutrients essential for microorganisms and plant growth, such as Ca, Mg, Bo, Cu, Fe, Mn, Mo and S (Nguyen et al. 2016). Other observations were performed in greenhouse condition to evaluate the efficacy of poultry ES powder, snail shell powder, dried-leaf powders of moringa and *Citrus aurantium* in comparison with recommended dose of furadan on growth of *C. aurantium* infected with *Meloidogyne incognita*. Results have revealed that all the tested materials significantly enhanced plant growth parameters of *C. aurantium* as well as diminished nematode *M. incognita*. Among the individual treatments, poultry ES powder (5 g/ plant) gave the maximum increase and ranked first in reducing the number of nematode galls (75.7%), females (79.2%), and egg masses (78.9%), followed by *Citrus aurantium* dried-leaf powder application. It was also observed that combined treatment of poultry ES powder plus furadan was over headed to that of *C. aurantium* dried leave power plus furadan in improving plant growth parameters (Helal et al. 2015).

Conclusion

The present experiment was done for sustainable administration of chicken egg shell waste through a simple approach and can be applied for plant cultivation. The results unveiled that eggshell in its powdered form and in combination with CFPH and VC can ameliorate the production of capsicum plants. Therefore, combination of ES, CFPH and VC could enrich the soil with both micro and macronutrients and it proves to be another possible source of organic compost having potential for plant growth promoting ability for crops in both agricultural and horticultural fields.

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Conflict of interest The authors declare that there are no conflicts of interest associated with this study.

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