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ORIGINAL RESEARCH

Vermicomposting of cow dung amended with eggshell powder: Possible roles of eggshell powder on the growth models of *Serendipita indic*, wheat growth and performances and soil enzymes activity

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Abstract

Purpose Chicken eggshell as a byproduct of the egg product industry makes serious problems to the environment. The aim of this study was to evaluate the effect of eggshell as a potential option in supplementing of the organic matter and improvement of soil conditions in the pot culture and the area.

Method Two pot experiments were carried out to estimate the effects of CD and CDES vermicomposts provided from cow dung and cow dung + eggshell's powder, respectively, on symbiotic relationships of *Serendipita indica* with wheat and soil enzyme activities tested under sterilized and non-sterilized soil conditions, respectively. Two in vitro experiments were also conducted to test the effects of eggshell extract and humic and fulvic acids extracted from CD vermicompost on *S. indica* growth.

Results CDES vermicompost improved soil enzyme activities of urease, phosphatase and invertase. Root colonization of wheat with multiple species of mycorrhiza and facultative symbiont *S. indica* was improved by CDES. *S. indica* growth was induced by eggshell extract. Humic and fulvic acids increased in *S. indica* mycelia mats dry yield. Results revealed when cow dung was amended with eggshell powder provided a better condition for earthworm growth and enhancing the colonization percentage of wheat root with indigenous mycorrhizal fungi. CDES vermicompost caused the increase in soil basal respiration and soil enzymatic activities.

Conclusion Eggshell powder growth promoting effect can be attributed to the biologically active compound that exists in eggshell extract.

Keywords Chicken eggshell, Soil enzyme, Fulvic acids, Humic acid, *Serendipita indica*, Vermicompost

Introduction

Interest in applying organic fertilizer in the agricultural systems has been growing in recent decades. Agricultural based intensive cropping with higher amounts of

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chemical fertilizer input has gradually declined the quality of soil conditions and its fertility. There are considerable agricultural organic wastes that can be considered as an important soil conditioner and soil additive to restore the fertility of soil in the area's. There is enough evidence that clearly proves the advantages of using organic manure in improving the fertility and conditioning of the soil. Animal waste as a suitable nutrient source for plants can be used to improve the physical and chemical properties, healthy and fertility of a soil (Franzluebbers 2002). There has been a very long history of the addition of various agricultural organic wastes to improve the soil conditions. Vermicomposting can be a sustainable strategy for better management of organic fraction of solid wastes and to transforms organic waste into organic fertilizer that would be suitable for sustainable agriculture (Liégui et al. 2021; Hosseini Jafari and Zarea 2021).

Chicken eggshell as a byproduct of the egg product industry makes serious problems to the environment too as a waste. Shuhadah et al. (2008) reported that in the U.S., about 150,000 tons of eggshells are discarded and disposing in landfills. Chicken eggshell is an excellent source of calcium carbonate (95%). Eggshell is rich in certain nutrient sources. Although calcium carbonate comprises 95% of the eggshell, as compared with calcium carbonate ($CaCO_3$) mineral, density of eggshell is lower (Hassan and Aigbodion 2015). Proteins, polysaccharides, and sulfates are the other nutrients present in eggshell waste (Hussein et al. 2011). Organic material comprises 5% of the eggshell (Hussein et al. 2011).

Soil ecology and biology can be affected by the condition of soil. Vermicompost has been reported to improve the health and nutrient status of soil (Orozco et al. 1996; Adhikary 2012; Xu and Mou 2016). Addition of vermicompost in soil has been shown to enhance the N-fixing microorganisms (Mackay et al. 1982) and enzymatic activities, too (Lavelle and Martin 1992). Studies showed that soil amended with vermicompost has been benefited crops (Mba 1996; Atiyeh et al. 2000b and Singh and Varshney 2013).

Decomposition of various organic matters by using various earthworms can be affected by several factors. Suchas, Temperature, moisture content and type of substrate materials (feeding material) can affect the growth of *E. fetida* (Edwards et al. 1998; Gunadi et al. 2002). The pH of material has been considered as the most important factor affecting survival of earthworms. pH values beyond optimal ranges, too acidic or too alkaline, C:N ratio of the substrate material are among the factors that affect the earthworm growth and survivals. The C:N ratio of 25 have been reported as suitable values by Ndegwa and Thompson (2000).

Serendipita indica, previously known as *Piriformospora indica*, (Weiß et al. 2016), belongs to a group of plant growth promoting microorganisms, in which the fungus is characterized with diverse plant growth promoting properties and has attracted attention worldwide. Facultative symbiont *S. indica* is an axenically cultivable fungi with plant growth promoting effects on host plants (Mohammadi Goltapeh et al. 2013). Published studies have been shown that *S. indica* successfully resulted in improving the growth of diverse plants species tested under non-stress and stress conditions (Xu et al. 2018; Gill et al. 2016; Zarea et al. 2012, 2013, 2014; Franken 2012; Varma et al. 2012; Waller et al. 2005; Xu et al. 2018).

Agricultural based intensive cropping with higher amounts of chemical fertilizer input has been gradually declined the quality of soil fertility and soil conditions. There are considerable agricultural organic wastes that can be considered as an important soil conditioner and soil additive to restore the fertility of the soil. The objective of this study was to evaluate if addition of eggshell

powder in cow dung can improve the chemical composition of the resulting vermicompost. The cow dung (CD) and cow dung + eggshell powder (CDES) were used in the preparation of organic compost (vermicomposting). Eggshell powder was applied to the cow dung at 1% dry weight. Besides, two pot experiments were conducted to evaluate the effect of two resulting vermicompots (CD and CDES), too, on soil enzyme activities with non-sterilized soil on wheat root-*S. indica* symbiosis in sterilized soil. An *in vitro* experiment was then carried out to elucidate the effect of eggshell powder and eggshell extract on *S. indica* growth also. In the fourth experiment, the effect of humic acids (HA) and fulvic acids (FA) extracted from cow dung vermicompost were evaluated on the growth of *S. indica* as well.

Material and methods

Experiment 1: Preparing organic compost by vermicomposting

Cow dung (CD) and cow dung + eggshell powder (CDES) were used in the preparation of organic compost by vermicomposting under greenhouse conditions using *Eisenia foetida*. To obtain eggshell powder, particles of eggshells were first dried out completely. Then microwave-dried at 200℃ for 4 minutes. After the drying process, eggshell particles were grinded, using mortar and pestle. Eggshell powder was applied to the cow dung at 1% dry weight. Cow dung was procured from the dairy cow farm of Ilam University, Ilam, Iran. The cow dung was used as feed for earthworms without any bedding materials. Initial chemical composition of the cow dung contained total nitrogen of 0.17%; total phosphorus of 38.31 ppm; potassium (K_2O) of 200.37 ppm, C of 7.84 % and pH of 7.82. Earthworms (*Eisenia foetida*), used for vermicomposting, were maintained on cow dung. To avoid volatile toxic substances, cow dung was turned every 24h for 14 days. After 14 days, eggshell powder was added and then mixed into cow dung at 1% dry weight (w/w). Known weights of 35 earthworms (8.68±0.45 g) were introduced in each plastic pot measuring 0.35 m× 0.25 m× 0.2 m (length × width × depth). All pots cultures were kept in a greenhouse at 25±3℃. CD and CDES were vermicomposted, using *E. fetida*. Three pots were used per each treatment. Cow dung alone and mixed with eggshell powder were the two treatments. Statistical analysis (t-Test) is performed for earthworm growth and chemical characteristics of vermicomposts. Moisture of the feed was maintained at about 70-80% (w.b.) by spraying the surface with adequate tap water every 2 days. Ndegwa et al. (1999) suggested that 750 g-feed kg^{-1} worm day^{-1} is the most favorable feeding rate. Based on that, each of the pots received a feeding rate of 6.42 g feed day^{-1}. At the end of the vermicomposting process (35 days), the total amount of feed that was given to the earthworms were 179.97g Pot^{-1}. After 5 weeks, total earthworm population, earthworm biomass, organic carbon and nutrient contents of N, P, K, pH, C and C:N were analyzed. Chemical composition of the produced vermicomposts was then analyzed. Organic C, total nitrogen (N), total phosphorus (P_2O_5), water soluble potassium (K2O), C/N and pH were analyzed after 5 weeks. pH was measured according to the method described by Erhart and Burian (1997). Humic and fulvic acids were extracted using the classic alkali/acid fractionation procedure (Valdrighi et al. 1996).

Experiment 2: Effect of CD and CDES Vermicomposts on association of wheat plants with *Serendipita indica*

A greenhouse study was setup for utilization of two obtained vermicomposts prepared from cow dung (CD) and cow dung + eggshell powder (CDES) on symbiotic

relationships of *S. idica* with wheat plant in terms of total root colonization percentage and chlamydospore formation. Treatment consisted of adding vemicompost (CD and CDES) and no addition to soils served as control treatment. Two types of produced vermicomposts (CD and CDES) were inoculated with *S. indica* spores and then were thoroughly mixed to the soil at 1% by dry weight. 100 spores 100 g vermicompost^{-1} were applied to the CD and CDES. Fungal spores applied in this study prepared from a liquid culture of *S. indica*. The culture of *S. indica* was obtained from Professor Ebrahim Mohammadi Goltapeh (Tarbiat Modares University, Tehran, Iran). The *S. indica* were grown and maintained on a solid medium in 80-mm Petri dish supplemented with 10 g L^{-1} agar. 5 agar discs (10×10 mm) were punched out and transferred to a 500 ml Erlenmeyer with 100 ml of Kaefer medium (10% glucose). Cultures were grown at 28°C on a rotary shaker at 120 rpm for 14 d. To dislodge the spores, 1.5 ml Tween 20 was added to the Erlenmeyer flasks. Flask cultures were shaken (at 200 rpm) vigorously, overtaxing and grinding for 10 min. Flask culture gave the yield of 4.28×10^3 spore. Soil used for pot culture was collected from crop land at the 0-30 cm depth of soil. Collected soil was sterilized at 120 °C for 20 min. 10 g of each provided vermicomposts kg soil^{-1} (on dry weight basis) was mixed thoroughly with sterilized soil. Autoclaved soil was used as control treatments. The experiments were conducted under greenhouse conditions. Experiment was laid out in Randomized complete block design (RCBD) with four replications for each treatment. Wheat seeds were surface sterilized by immersion in 98% ethanol for 30s, and followed by 2% sodium hypochlorite solution for 4 min. Seeds were then washed thoroughly with distilled water. Surface sterilized wheat seeds (*Triticum aestivum* L.) of cultivar Pishgam were sown at the rate of 10 seeds pot^{-1}. Pots were irrigated at 60 percent field capacity. Pots at the tillering growth stage received 1 g N pot^{-1} and 0.25 g P per pot^{-1}. Fertilization was made three times, at tillering, stem elongation and grain filling stages. The control pots were received the same amount of fertilizers. *S. indica* colonization rate was checked 30 days after the sowing time. Methods previously described by Phillips and Hayman (1970) and Kumar et al. (2009) with some minor modifications were followed to determine the fungal colonization percentage. 10 root samples taken randomly from 3 seedlings from each pot were softened by heating in 10% KOH solution for 15 min. Softened root samples were then acidified in 1 M HCl solution for 10 minutes. Samples were then rinsed in distilled water three times. Finally, root samples were stained by simmering in 0.02% trypan blue overnight. To remove excess stain, samples were stained with 50% lactophenol for 2 h. Total root colonization percentage and chlamydospore formation were determined under a light microscope.

Experiment 3: Effect of eggshell extract on *S. indica* growth

In vitro experiments were conducted to evaluate the potential of chicken eggshell extract on *S. indica* growth and fungal mycelia mats yield. Eggshell powder was prepared as mentioned previously. 1-g of eggshell ground to pass through a 1-mm sieve, was digested with 6 ml H_2SO_4 solution (1:6 w/v) and shook constant at 180 rpm for 72 h at room temperature. To prepare H_2SO_4 solution, 4 mL concentrated sulfuric acid was slowly added to 60 mL distilled water. Applying higher concentration of H_2SO_4 solution to eggshell resulted in the burning of eggshell powder (Fig. 1A-C). Eggshell extract was collected by centrifugation at 13000 rpm for 10 min. Soluble supernatant (eggshell extract) was resuspended in 1 N KOH solution. Eggshell particles concentrated at the bottom of the centrifuged tube (eggshell

pellet) were acidified with 6 ml H_2SO_4 solution. *S. indica* was grown on a solidified medium (1.5% agar) and in liquid broth medium supplemented with 0.1% eggshell powder (w/v), 0.1% eggshell extract and 0.1% eggshell pellet (concentrated eggshell particles after centrifugation). The medium supplemented with 1 ml L^{-1} H_2SO_4 was used as control treatment. The medium composition (g L^{-1}) was as follows: 5 malic acid, 0.5 yeast extract, 0.2 $MgSO_4$, 0.016 $FeSO_4$, 0.5 K_2HPO_4, 0.01 $CaCl_2$, 0.2 NaCl and 15 agar. The pH of the medium was adjusted to 6.8 with KOH. Sterilization was done at 120℃ for 20 min. Fully-grown fungi agar plug (10×01 mm) was removed from the leading edge actively growing colonies and then inoculated in 8-cm diameter Petri dishes, containing cultural medium. The medium supplemented with 1 ml L^{-1} H_2SO_4 was used as control treatment. Each treatment had five replications. Cultures were incubated at room temperature in the dark for 12 days. Radii of the fungal colonies were measured according to the method described by Zarea et al. (2012). 5 agar discs (10×10 mm) from the leading edge actively growing colonies were punched out and transferred to a 500 ml Erlenmeyer flukes with 100 ml of the medium as mentioned previously. Liquid broth cultures were incubated in a shaker at 25℃ and at110 rpm for 4 weeks. After 4 weeks, the mycelial mats were harvested from the liquid media. The mycelial mats were dried at 70℃ and then weighed.

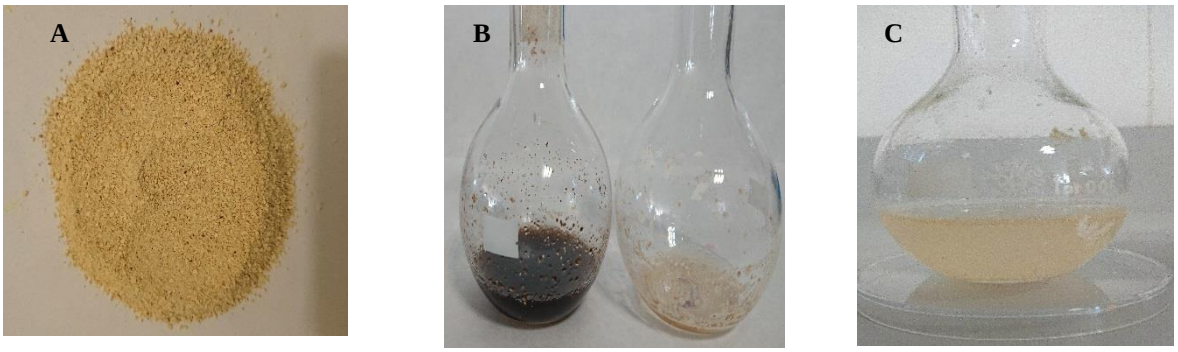

Fig. 1 Processing of eggshell extraction

Note: A: grinded eggshell. **B**: 1-g amount of eggshell powder was digested with 6 ml H_2SO_4 solution (1:10 w/v) (**B**, left) and with 6 ml H_2SO_4 solution at the ratio of 6:1 w/v (**B**, right). **C**: resulting digested eggshell provided from 1 g eggshell powder digested with H_2SO_4 solution (1:6 w/v) for 72 h, shaking constant at 180 rpm for 72 h at room temperature.

Experiment 4: Effect of humic and fulvic acids on *S. indica* growth

An *in vitro* experiment was conducted to elucidate how humic acid (HA) and fulvic acid (FA) extracted from CD affect *S. indica* growth. Published studies provided clear enough evidence that large quantities of humic substances are produced during the vermicomposting process. The effect of humic and fulvic acids on *S. indica* growth was assayed under both solid and liquid cultures. Using Methods of Valdrighi et al. (1996) and Vallini et al. (1990) with some minor modification, based on alkali/acid fractionation procedure, were followed to extract humic and fulvic acids. Briefly, HA from CD vermicompost was extracted as follows: vermicompost was first digested with 100 mM KOH solution for 24 h

at room temperature in the ratio of 1:10 (w/v). The extracted solution was then separated from the insoluble sediment by centrifugation at 13000 rpm for 15 min. Soluble supernatant was then filtered and acidified at pH=2 with concentrated H_2SO_4 (98%) and maintained in this condition for 24 h at room temperature. Humic acids were finally collected by centrifugation at 13000 rpm for 15 min. 0.73 g of humate from 1 kg of vermicompost was extracted. Humic acid gel was resuspended in 100 mM N KOH solutions. Soluble supernatant (soluble fulvic acids, FA) was neutralized with KOH. *In vitro* experiments were performed to evaluate the efficacy of supplementation of various levels of HA and FA extracts to test their effectiveness on *S. indica* growth under both solid and liquid cultures. The experiments, replicated five times, were carried out in completely random design with five HA and FA levels (0, 250, 500, 1000, 2000 and 4000 µl in 1 L^{-1} culture medium). Sterile solid and liquid *in vitro* cultures of *S. indica* supplied with different concentrations of HA and FA were established. One fungal agar plug (10 mm), obtained from freshly grown culture plates of *S. indica*, were inoculated in the centre of solidified broth medium agar plates (8-cm diameter Petri dishes). Culture medium plates were incubated in dark at room temperature (25℃). Growth of *S. indica* was evaluated in terms of radial expansion on the culture medium plate. Additionally, the density of fungal mycelium in liquid culture supplemented with various concentrations of HA and FH was evaluated. 100 ml medium, supplemented with various levels of HA and FA, were poured into 500 ml Erlenmeyer flasks and then autoclaved. 5 fungal disc (10 mm) of S. *indica* was transferred into the media. Each treatment consisted of 5 replicates. Cultures were incubated in an incubator shaker at 25℃ by shaking at 110 rpm for 4 weeks. Fungal biomass was determined after incubation. Dried mycelia mats were measured after drying at 70℃.

Experiment 5: Effect of CDES and CD vermicomposts on soil enzymes activity and wheat growth and yield

The objective of this study was to investigate the effect of the amendments with CDES and CD vermicomposts on wheat growth and enzyme activity in soil. Vermicompost treatments consisted of no addition (control treatment) and addition of CDES and addition of CD vermicomposts at a rate of 10g kg^{-1} soil. The original soil for the greenhouse pot experiment was collected from the top surface (0-30 cm) of farmland soil. The experiment was carried out in RCBD with 3 treatments, having 3 replications. Plastic pots were filled with 17 kg of farmland soil. Wheat seeds were first surface sterilized by immersion in 98% ethanol for 30s, and then followed by 2% sodium hypochlorite solution for 4 min. Seeds were then washed thoroughly with distilled water. Surface sterilized wheat seeds (*Triticum aestivum* L.) of cultivar Pishgam were sown at the rate of 10 seeds pot^{-1}. The pots were then thinned to 5 germinated plant per pot. Pots were irrigated at 60 per cent field capacity. The pots at the tillering growth stage received 1 g N pot^{-1} and 0.25 g P pot^{-1}. Fertilization of pot cultures were made three times, at tillering, stem elongation and grain filling stages. The control pots received the same amount of fertilizers. Also, at the stem elongation stage, fresh soil samples were collected from 0-15 cm in each pot to analyze the soil enzyme activities. To analyze the enzyme activity of soil, the method described by Tabatabai (1994) was followed. The soil phosphatase activity and urease activity were measured by determination of the phenol release and NH_4^+ release, respectively.

Soil samples were incubated with sucrose for 4 h at 35 °C and then the amount of glucose released was determined as invertase activity. Soil respiration was measured at stem elongation stage based carbon dioxide trapping in NaOH solution. CO2 evolution was estimated by titrating NaOH with 0.05 M HCl. Plants were harvested at the grain maturity stage. At harvest, grain yield, 1000-grain weight and shoot plant fresh and dry weights were recorded. 3 plants were randomly selected from each pot for mycorrhizal colonization fungi determination.

Statistical analysis

All in vitro experiments were conducted in completely randomized design. Pots culture experiments laid out in complete randomized block design. The data was analyzed by ANOVA using the SAS software package (SAS Institute 2000). Least significant difference (LSD) was used to test the significance of the differences between or among the treatments at $P < 0.05$.

Results and discussion

The chemical characteristics of vermicomposts derived from cow dung (CD) and cow dung + eggshell powder (CDES)

Chemical composition of vermicomposts produced from CD and CDES were analyzed after 35 d. The chemical analysis, C, N, P, K, C, C:N and pH, is listed in Table 1. No significant differences were found between CD and CDES vermicomposting with respect to K and P. Total N content in CD vermicompost was significantly ($P < 0.05$) higher than in CDES vermicompost (Table 1). In the present study, pH content in CDES vermicompost was significantly higher ($P < 0.05$) than in CD vermicompost. CDES vermicompost had higher C than CD vermicompost. The C:N ratio in CDES was higher than in CD (Table 1). Vermicompost from the CD showed lower Humic and Fulvic acids content (Table 1). Adding eggshell to cow dung increased the Humic and fulvic acids content of vermicompost (Table 1). Estimated final biomass and biomass gain of earthworms were significantly ($P < 0.05$) higher in the CDES vermicompost than in the CD vermicompost (Table 2). The total amount of vermicompost produced was up to 130 g. The total amount of vermicompost produced from CD and CDES was not significantly different (Table 2). Nutrient content of vermicompost can be determined by various factors. Higher content of nutrient in vermicompost can be attributed to the higher rate of organic mineralization that is accelerated by earthworm's activity. Addition of eggshell powder into cow dung as an additive had no significant effect on mineral nutrient contents of K and P in the final product. Eggshell powder seems to have no effect on mineralization by earthworm's activity. Mainly the final Earthworm biomass in the resulting byproduct vermicompost from CDES was higher than in vermicompost produced from CD. These significant differences among CD and CDES treatments could be caused by the quality and chemical prosperity of eggshell that was mixed to cow dungs. *Eisenia fetida* is the most eurythermal epigeic earthworm (Reinecke et al. 1992) preferring neutral to slightly alkaline pH (Pramanik et al. 2007). In this study, eggshell-amended cow dung was slightly alkaline. The pH in CD (6.40) and CDES (7.33) vermicasts was significantly different. The pH was slightly alkaline in the CDES vermicompost (7.33). Although the pH value of the vermicompost has been reported to decrease due to organic acids and CO_2 produced during microbial metabolism (Hartenstein and Hartenstein 1981), it is substrate dependent and dynamic (Ndegwa et al. 2000).

Table 1 Statistical analysis (t Test) of final chemical composition

Chemical properties		Mean	Standard Deviation	p-value
N (%)	CDES	1.07	0.01	0.056*
	CD	1.32	0.11	
P (mg kg^{-1})	CDES	26.21	1.82	0.2059^{ns}
	CD	23.78	2.09	
K (mg kg^{-1})	CDES	123.88	12.58	0.6024^{ns}
	CD	135.89	32.85	
pH	CDES	7.11	0.015	0.0142**
	CD	6.40	0.151	
C (%)	CDES	58.2	6.30	0.0547*
	CD	46.17	5.04	
C:N	CDES	54.42	10.76	0.0567*
	CD	35.1	3.68	
Humic acid (mg g^{-1})	CDES	0.73	0.04	0.0279**
	CD	0.62	0.01	
Fulvic acid (mg g^{-1})	CDES	0.69	0.015	0.0015**
	CD	0.59	0.016	

^{ns},* and ** : no significant and significant at the 1% and 5% levels of probability, respectively.
CD: final vermicompost provided from cow dung
CDES: final vermicompost provided from cow dung +eggshell powder

Table 2 Statistical analysis (t Test) of *Eisenia foetida* growth

Parameters		Mean	Standard Deviation	p-value
Initial biomass (g $worm^{-1}$)	CDES	0.242	0.0035	0.3353^{ns}
	CD	0.239	0.0038	
Final biomass (g $worm^{-1}$)	CDES	0.30	0.021	0.0556*
	CD	0.28	0.039	
Biomass gain (g $worm^{-1}$)	CDES	0.054	0.012	0.0563*
	CD	0.038	0.011	
Quantity of vermicompost (g)	CDES	134.33	5.0	0.1165^{ns}
	CD	130.25	2.5	

^{ns},* and ** : no significant and significant at the 1% and 5% levels of probability, respectively.
CD: final vermicompost provided from cow dung
CDES: final vermicompost provided from cow dung +eggshell powder

Eggshell is an excellent source of calcium carbonate (95%). Calcium carbonate present in eggshell powder might result in reducing the acidity of the cow dung during vermicomposting processes. Microbial activity during vermicomposting can affect the rate of mineralization of organic N, resulting in increasing the amount of N in the final vermicompost. In the present study, lower amounts of N content of CDESH vermicompost might not be attributed to the microbial activities. Microbial activity during vermicomposting in eggshell-amended cow dung was higher than cow dung alone (data not shown). Earthworm activity results in fragmentation of organic substrate, increases the rate of mineralization and stimulates microbial activities, converting the substrates into humus-like substances (Atiyeh et al. 2002). Microbial activities during vermicomposting affects the rate of decomposition, C: N ratio rate affects and the rate of decomposition. Although adding eggshell increases C:N ratio, it stimulates microbial activity during the vermicomposting process.

Effects of CDES and CD vermicomposts on wheat growth and root infection by *Serendipita. Indica*

Results showed that the wheat growth and yield were affected significantly ($P < 0.05$) by the application of vermicomposts into soil. The fresh and dry weights of shoots, grain weight and grain yield were increased by addition of CDES and CD vermicomposts, although the stimulation was greater in CDES vermicompost (Table 3). Both vermicomposts stimulated root colonization (Table 3). Higher root colonization and chlamydospores formation were observed in plants grown in soils amended with CDES vermicompost (Table 3). Fig. 2 shows microscopic view of the fungal colonization in 14-d old and 30-d old wheat roots, grown in soils amended with CDES vermicompost. Root colonization and chlamydospores formation increased in CDES vermicompost-amended soil's (Fig. 2).

Table 3 Agronomic traits and colonization percentage of *S. indica* of wheat in response to application of cow dung vermicompost (CD) and cow dung + eggshell vermicompost (CDES)

Treatment	Fresh dry matter	Dry matter	1000-grain weight	Grain yield pot^{-1}	Colonization
CDES	29.1 (±1.0)a	8.4 (±0.94)a	31.1 (±1.85)a	5.9 (±0.56)a	65.50 (± .93)a
CD	26.1 (±1.1)b	7.6 (±1.22)a	29.5 (±1.65)b	5.3 (±0.62)b	51.5 (±1.32)b
Control	17.7 (±1.3)c	5.1 (±1.65)b	27.6 (±1.2)c	3.3 (±0.24)c	-
LSD	1.49	0.82	1.46	0.44	3.9

Means in each column followed by same letter don't show significantly difference (n = 4).

Phytohormones such as gibberellins, cytokinins and IAA have been reported to extract from vermicomposts (Arancon et al. 2006). Vermicompost as soil conditioner has been reported to increase microbial populations and activity through providing micro sites (Atiyeh et al. 2000a). Improvement of physical properties of soil can also influence the soil microbial activities. Vermicompost plays an important role in physicochemical properties of soil. Mycorrhizal colonization has been reported to significantly be promoted by humic acid rich vermicompost (Maji et al. 2017). Based on statistical analysis, CDES vermicomposting were more effective in plant growth performances than CD vermicomposting (Table 4). Eggshell is composed of macro- and micronutrients such as P, K, Ca, Mg, Zn and Cl (Makkar et al. 2015; Barker and Pilbeam 2007) that are essential for plant growth. Besides, eggshell contains uronic acid and amino acid (Hincke et al. 2012) with biologically active compounds such as sialic acid (Burley and Vadehra 1989) that can be effective in promoting effect on soil microorganism growth and proliferation. Phytohormones can be efficiently affect the plant responses to the *S. indica*. It has been reported that phytohormones play vital roles in interactions of plants with microbes such as *S. indica* (Xu et al. 2018). Phytohormones such as salicylic acid are involved in the interaction of *P. indica* with higher plant species (Xu et al. 2018). Although eggshell contains toxic elements such as Al, Pb Hg and Cd, levels of them are very low as are levels of P, Mg, N, F, Se, Cu, V, B, Fe, Zn, and Cr. However, eggshell application as a by-product in agriculture to the soil has been studied a little as a source of nutrient to plants.

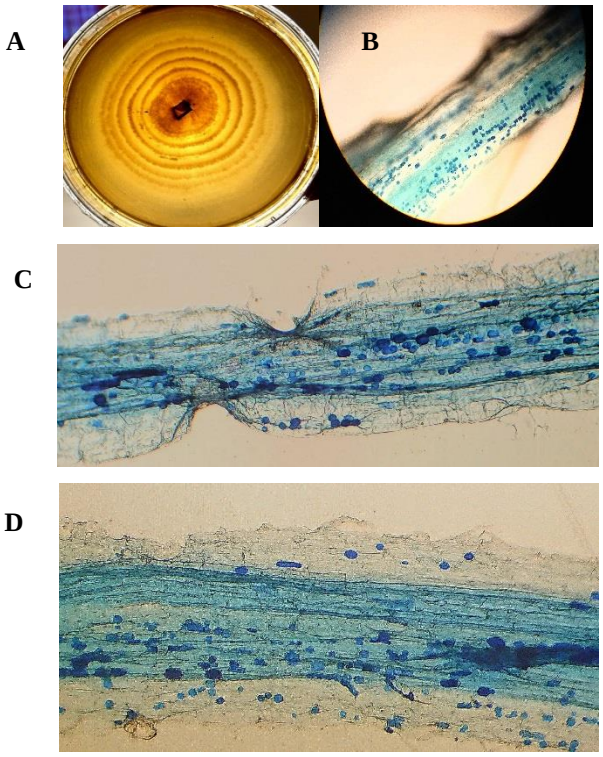

Fig. 2 The growth of *S. indica* in modified Kaefer medium and representative microscopic view of the *S. indica* colonization

A: Representative **t**he colony morphology of an individual disc colony of *S. indica* growing on Petri dishes containing modified Kaefer medium (10% glucose); **B and C:** representative microscopic view of the fungal colonization in 14-d old and 30-d old wheat roots, respectively, grown in soils amended with CDES vermicompost; **C:** Microscopic view of the fungal colonization in 30-d old wheat roots planted in soil amended with cow dung vermicast alone (CD). (**Note**: Plant root stained with trypan blue).

Effect of eggshell extraction on *Serendipita indica* growth

Fig. 3 is representative of the colony morphology of an individual disc colony of *S. indica* growing in Petri dishes containing eggshell powder (0.1%) and eggshell extract. Addition of eggshell powder (1%) in solid culture medium improved the growth of *S. indica* in terms of fungus radial growth (Fig. 4A). Cell dry weight was significantly affected by eggshell extraction. In this study, the presence of eggshell extraction in the medium produced a higher number of mycelia mats (0.109 g 100 ml^{-1}) (Fig. 4B). The linear growth and dry biomass of *S. indica* was affected by eggshell extract. Although linear growth was accelerated by adding eggshell powder in broth medium, the mycelia mats yield was increased with eggshell extracts introduced to broth medium at concentration of o.1% L^{-1}. This may be related with the stimulatory effect of eggshell extraction. Eggshell extraction may contain a biologically active compound that affects *S. indica* growth and colonization. Previous study indicated that the non-edible by-products of chicken eggshell, consisting of outer and inner membrane and calcified shell, contain small amounts of lipid, carbohydrates and proteins. It has been also suggested that hormone-like activity is present in eggshell powder

(Rovensky et al. 1994), this might play a role in the effects on *S. indica* growth. Sialic acid has been reported to be found in eggshell inner and outer membranes (Burley and Vadehra 1989). Nakano et al. (2003) reported the presence of amino acid, glycine and alanine, uronic acid, proline and hydroxyproline in chicken eggshell. Nakano et al. (2001) also found uronic acid in chicken eggshells. Decalcified eggshell contains alactosaminoglycans and hyaluronic acid (Nakano et al. 2001).

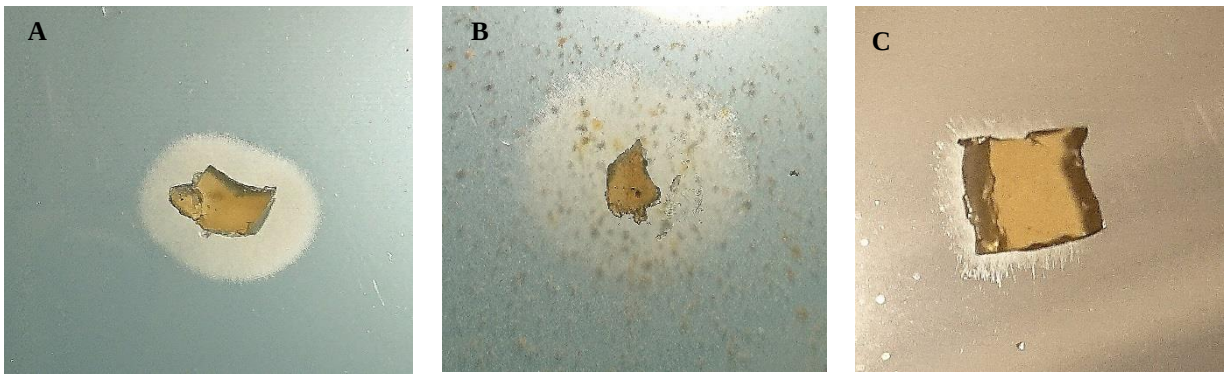

Fig. 3 Cultivation of *S. indica* on solidified broth culture supplemented with 0.1% eggshell extract (**A**), 0.1% eggshell powder (**B**) and eggshell pellet (**C**) at 25°C for 5 days

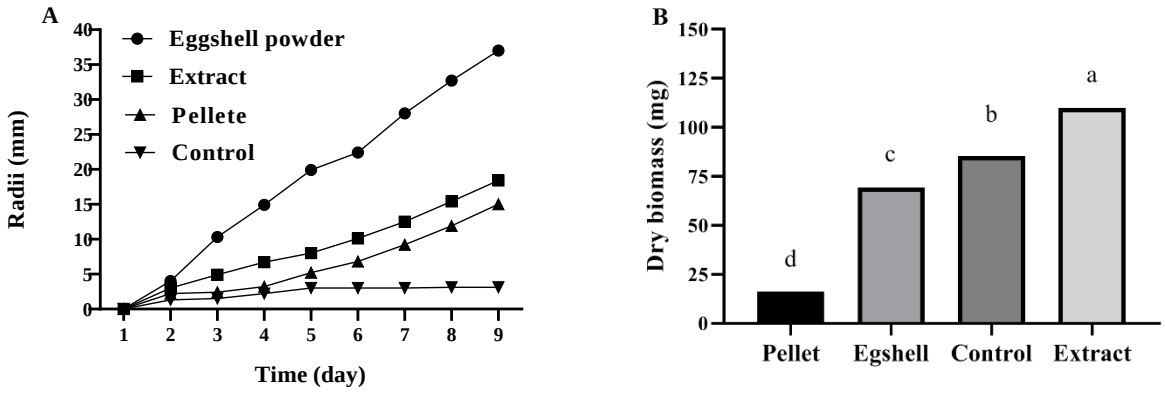

Fig. 4 Effect of eggshell extraction on *S. indica* radial growth and mycelia mat yield

A. Calculated mean radii of *S. indica* on solidified broth medium measured on four perpendicular axes bisecting the center of the colony. (**Note:** The diameter of the fungus was divided by its average growth. *S. indica* was grown on the solidified broth cultures supplemented with 0.1% eggshell powder (w/v), 0.1% eggshell extract and 0.1% eggshell pellet (concentrated eggshell particles after centrifugation). $P < 0.05$. Values are mean ± SE (n = 5). **B**, The mycelia mats (dry biomass) yield of *S. indica* in medium supplemented with 0.1% eggshell powder (w/v), 0.1% eggshell extract and 0.1% eggshell pellet. (**Note:** The mycelia mats were harvested from the liquid media after 4 weeks). $P < 0.05$. Values are mean ± SE (n = 5).

Effect of humic and fulvic acids extracts on *Serendipita indica*

Addition of HA to the broth medium at different rates influenced the linear growth of *S. indica*. Fig. 5 is representative of the colony morphology of an individual disc

colony of *S. indica* growing in Petri dishes containing HA and FA in comparison with control. According to the results obtained in this study, the higher (1000, 2000 and 4000 µl L^{-1}) rates of HA had the inhibitory effect on linear growth of *S. indica* (Fig. 6A). Additionally, addition of HA to the liquid medium higher than 500 µl L^{-1} significantly decreased the yield of mycelia mats (Fig. 6B). The yield of mycelia mats at concentration of 200, 500 and 1000 ppm HA were increased. Applying FA to the broth medium declined the linear growth of *S. indica* (Fig. 7A). The mycelia mat weight showed a significant increase with increasing FA concentration (Fig. 7B). Humic substances have been demonstrated as biostimulator (Shah et al. 2018). Humic acid possesses a biological (phyto-hormone-like) activity (Fike et al. 2001; Nardi et al. 2000, 2002). Studies have shown that humic substances can affect enzymatic reaction, protein synthesis, nucleic acid synthesis and inhibiting or increasing different enzymes (Khaleda et al. 2017; Shah et al. 2018). Besides plants, soil microorganisms also responses to HA have been found positive (Pathma and Sakthivel 2012). Humic acid as a source of nutrients (amino sugars, organic acids, reducing sugars and peptides) may promote growth and proliferation of microorganisms (Sellamuthu and Govindaswamy 2003). Microbial biomass has been found to be increased due to carbonaceous substrates that are made available by the addition of humic acid (Blagodatskaya et al. 2014). In the present study, humic acid showed a stimulatory effect on *S. indica* growth. Despite the results obtained in the present study by humic acid in stimulating growth of *S. indica*, higher concentration of HA surpassed *S. indica* growth. Unfortunately, there is no data on *S. indica* by other authors that can be compared with the present results. Adding FA in broth culture increased the linear growth and mycelia mat yield of *S.indica*. This increase was significant when FA applied between 250 and 1000 µ l^{-1} L^{-1}. As compare with the controls, when 1000 ppm and more was added to the broth culture linear growth and mycelia mat yield tended to decline. To our knowledge, this is the first report that has elucidated the role of FA on *S. indica* growth. However, in an experiment, the toxicity effect of FA at higher concentrations on colony-forming units of bacteria and saprophytic fungi has been previously reported by Gryndler et al. (2005).

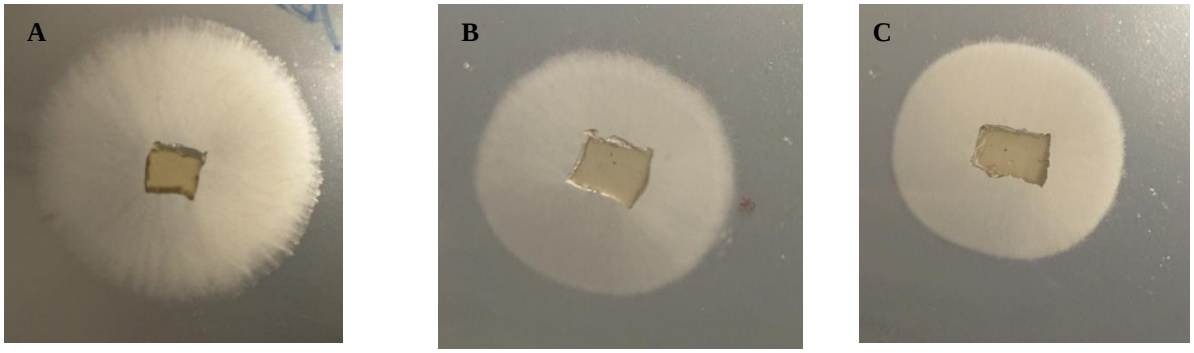

Fig. 5 The colony morphology of an individual disc colony of *Serendipita indica* growing on petri dishes containing seven concentration rates of Fulvic acids (**A**) and Humic acid (**B**) in comparison with the controls (**C**)

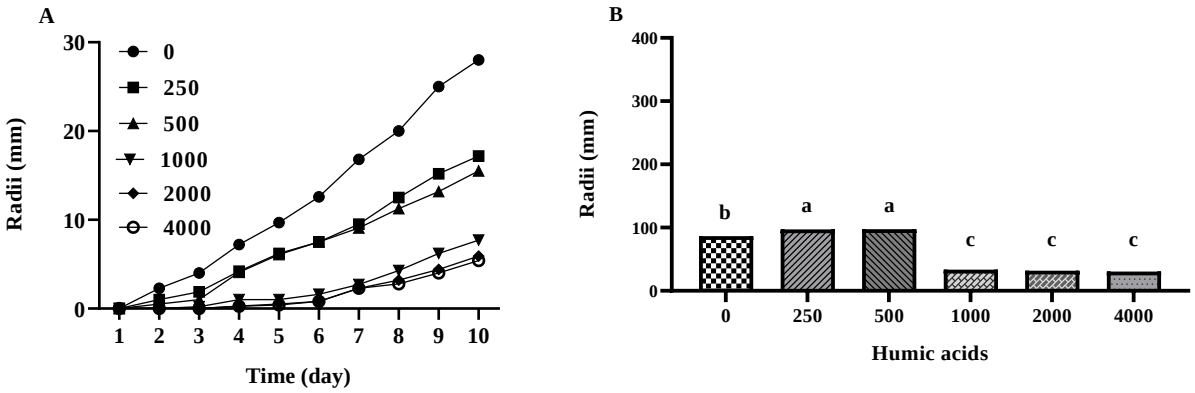

Fig. 6 Effect of different rates of humic acids extracts on *Serendipita indica* growth

A, Dose-response curves for the growth of *S. indica* on solidified broth medium amended with seven concentrations of humic acids (0, 250, 500, 1000, 2000 and 4000 ppm L^{-1}). (**Note:** The diameter of fungus was divided by its average growth on four perpendicular axes bisecting the center of the colony. $P < 0.05$. Values are mean ± SE (n = 5). **B**, The mycelia mats (dry biomass) yield of *S. indica* growing in medium at seven humic acid levels (0, 250, 500, 1000, 2000 and 4000 ppm L^{-1}). $P < 0.05$. Values are mean ± SE (n = 5).

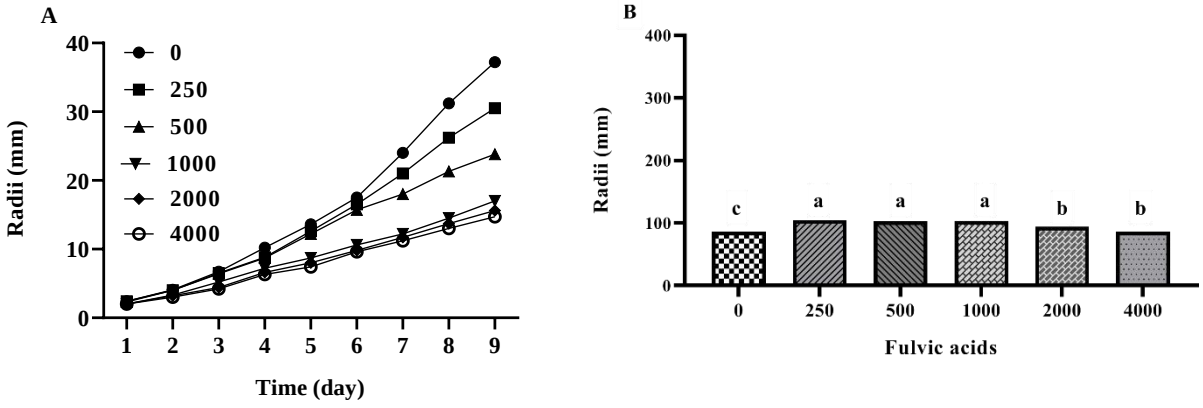

Fig. 7 Effect of different rates of fulvic acids extracts on *Serendipita indica* growth

A, Dose-response curves for the growth of *S. indica* on solidified broth medium amended with seven concentrations of fulvic acids (0, 250, 500, 1000, 2000 and 4000 ppm L^{-1}). (**Note:** The diameter of fungus was divided by its average growth on four perpendicular axes bisecting the center of the colony. $P < 0.05$. Values are mean ± SE (n = 5). **B**, The mycelia mats (dry biomass) yield of *S. indica* growing in medium at seven fulvic acid levels (0, 250, 500, 1000, 2000 and 4000 ppm L^{-1}). $P < 0.05$. Values are mean ± SE (n = 5).

Effect of chicken eggshell on soil enzymes activity and wheat growth and yield in non-sterilized soil

The effects of cow dung (CD) vermicompost and cow dung+eggshell (CDES) vermicompost added to soil at the ratio of 10 g kg^{-1} (1%) on plant fresh and dry matter accumulations, grain weight and grain yield are shown in Table 4. All wheat agronomic traits recorded were significantly improved by both vermicomposts as compared with control treatment (Table 4).

Based on statistical analysis, CDES vermicompost was more effective than CD vermicompost on plant growth performances (Table 4). As shown in Table 4, the second comparison can be between CD and CDES on soil enzyme activities (Soil respiration, Ureas, phosphatase and invertase) and mycorrhizal colonization rate. The CDES is significantly more effective than the CD.

There is enough evidence on the beneficial effects of Vermicompost for plant growth (Singh et al. 2006; Zucco et al. 2015; Mochache et al. 2021; Awadhpersad et al. 2021).

Chicken eggshell is composed of organic and inorganic materials. Eggshell contains macro- and micro-nutrients such as P, K, Ca, Mg, Zn and Cl (Makkar et al. 2015; Barker and Pilbeam 2007) are essential for plant growth and their yield (final products).

In the percent study, addition of CDES to the soil resulted in improvement of soil enzyme activity of urease, phosphatase and invertase, soil respiration and mycorrhizal colonization rates.

Soil physico-chemical conditions and soil microbial statues can be detected through soil enzyme activities (Sardans et al. 2008; Burke et al. 2011).

Various factors such as soil pH value, nutrient statues, C availability and soil moisture can affect the soil enzyme activities (Wang et al. 2009; Fernández-Calviño et al. 2010). Increased soil enzyme activities after CDES application may be due to increased available nutrients. Besides, eggshell contains uronic acid and amino acid (Hincke et al. 2012) with biologically active compounds such as Sialic acid (Burley and Vadehra 1989) that can be effective in promoting effect on soil microorganism's growth and proliferation.

Conclusion

The addition of soil amendment, eggshell powder, in the preparation of organic compost by vermicomposting affected the chemical composition of the resulting cow dung vermicomposts. These studies revealed when cow dung manure was amended with eggshell better conditions for earthworm. Vermicompost derived from cow dung + eggshell powder effectively enhanced wheat growth and performances, soil enzyme activity and symbiotic relationships of *S. idica* with wheat plant that can be attributed to the biologically active compound that exists in eggshell extract. Eggshell extract derived from eggshell powder enhanced S. *indica* growth that may confirm recent hypothesis. Thus, eggshell powder can be used as soil conditioner. However, additional doses of eggshell powder and long-term monitoring of its effects is also recommended.

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Compliance with ethical standards

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

Table 4 Effect of CD and CDES vermicomposts on agronomic traits, mycorrhizal colonization, soil respiration and enzyme activity

Treat-ment	Fresh dry matter	Dry matter	1000-grain weight	Grain yield pot^{-1}	Mycorrhizal colonization (%)	Respiration rate (μg CO_2-C soil g^{-1} day^{-1})	Urease (mg NH_4^+)	Phosphatase (mg phenol kg^{-1} h^{-1})	Invertase (mg glocuse kg^{-1} h^{-1})
CDES	29.3 (±1.0)a	7.5(±0.94)a	32.2(±1.85)a	4.7(±0.56)a	43.3 (±2.1)a	29.84(±0.73)a	55.6(±0.15)a	63.2(±0.16)a	36.7(±0.32)a
CD	22.1(±1.1)b	7(±1.22)b	30.6(±1.65)b	4.2(±0.62)b	37.7(±1.15)b	26.76(±0.43)b	48.7(±0.1)b	53.0(±0.07)b	32.0(±0.26)b
Control	19.9(±1.3)c	6.6(±1.65)c	28.9(±1.2)c	3.7(±0.24)c	31.8(±1.1)c	23.13(±0.90)c	33.5(±0.1)c	42.7(±0.06)c	24.0(±0.34)c
LSD	2.69	0.23	1.15	0.26	4.2	1.8	1.16	4.5	1.7

Means in each column followed by same letter don't show significantly difference (n = 4)

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