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Effects of Biochar on Yield of Chilli, and Soil Chemical Properties

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aims: To examine the effects of different doses of biocharon the yield of chili and soil chemical properties under the Samtenling condition which represents the wet-subtropical agro-climatic zone of Bhutan.

Study Design: Randomized Complete Block Design (RCBD) was used for the study with three replications and five treatments.

Place and Duration of Study: Agriculture Research and Development Centre (ARDC), Samtenling, Sarpang district located in southern Bhutan, between October 2020 and May 2021.

Methodology: Composite soil samples were collected before and after biochar addition and their chemical properties were analyzed at Soil and Plant Analytical Laboratory (SPAL), National Soil Services Centre, Thimphu. One hybrid variety (sv2319HA) of chili was used in all the treatments with five different biochar doses (No biochar addition, biochar addition @ 0.8 t acre⁻¹, 1.6 t acre⁻¹, 3.2 t acre⁻¹, and 4.9 t acre⁻¹). Growth and yield data of chili were collected and analyzed using the multivariate analysis of variance (MANOVA) at P = .05using R studio team (2021), version 1.4.1103. **Results:** The yield of chilli significantly differed (P = .03) under different doses of biochar but variations in growth were insignificant except the number of branches (P = .03). Overall, the yield of chilli increased from 0.3 t acre⁻¹ to 0.9 t acre⁻¹ with an increase in biochar rates from 0.8 t acre⁻¹ to 3.2 t acre⁻¹. Biochar addition of 3.2 t acre⁻¹ significantly increased the yield of chili (0.95 t acre⁻¹) as compared to control (0.346 t acre⁻¹). Soil pH, % nitrogen, available P (mg kg⁻¹), available K (mg kg⁻¹), CEC, % BS, and % organic matter increased with increasing quantities of rice husk biochar.

Conclusion: The different doses of biochar addition to the soil had a significantly positive impact on the yield of chili as it also improved the soil's chemical properties. The addition of 3.2 t acre⁻¹ of biochar may be recommended as per the study since the maximum yield of chilli was recorded in soil treated with biochar @3.2t acre⁻¹.

Keywords: Biochar; chilli; yield; growth; soil chemical properties.

1. INTRODUCTION

Biochar is a recent but underutilized soil technology. Biochar (charcoal or agri-coal) is a carbon (C) rich product derived from the pyrolysis of organic material at relatively low temperature (<700°C) [1], having potential use as an amendment to enhance soil properties and agricultural systems productivity. It is mainly composed of stable aromatic forms of organic carbon that do not degrade to CO₂ making it a relatively simple climate change mitigation method [2]. The application of biochar to soil could play a substantial role in mitigating greenhouse gas emissions and climate change effects [3]. The highly porous nature of biochar improves the soil water retention ability as well as plant available water (PAW) [4]. Studies indicated that the application of biochar improves crop production by increasing plant nutrient uptake [5] and improving the physical and chemical properties of soil such as water holding capacity, cation exchange capacity (CEC), adsorption of plant nutrients and soil pH [2.6]. Therefore, it enhances soil fertility [7] and sustains crop productivity [8,9] while it also reduces nutrient leaching loss [2].

Chilli is an important ingredient of Bhutanese dietary habits, consumed either as a vegetable or used as a food flavoring spice. In Bhutan, the average import and production of chilli from 2014-2018were1300.716 MT and 9,261 MT respectively with an average yield of 1.8 t acre⁻¹ [10]. The import restriction in recent years led to an increased need for domestic production. However, water and nutrient deficiency often limits crop growth and development as chilli is a high nitrogen feeder and sensitive to water stress [11,12]. Studies also reported that the soil fertility status in Bhutan is low to medium, and the mean pH value is acidic in nature (<5.6) which indicates the need to improve soil fertility through the application of various soil amendments [13]. The positive effects of biochar application on crop yields in comparison to unamended soil have been reported in various crops such as rice [14], maize [15], soybean [16], cassava [17], and chilli [18]. A study conducted by [19] showed that

biochar application increased the yield of maize and cabbage by 6.66% (12.31 t ha⁻¹ to 13.13 t ha⁻¹) and 7.57% (1.98 t ha⁻¹ to 2.13 t ha⁻¹) respectively. Further, [20,21] observed that biochar doses of 6-9 t ha-1provided the best economic yields in vegetable crops. Therefore, this study was conducted to examine the effects of biochar on soil properties and assess the influence of biochar on the growth and yield of chilli under the Samtenling condition which represents the wet-subtropical agro-climatic zone. In this experiment, we hypothesized that the biochar will significantly (I) increase the yield of chili, and (II) improves the soil's physical and chemical properties.

2. MATERIALS AND METHODS

2.1 Study Site

The study was conducted at Agriculture Research and Development Centre, Samtenling, Sarpang in 2020 and 2021. The experimental site falls under the wet-subtropical agroecological zone and is located (26° 54'30" N and 90° 25'55"E) at 375 masl with an average annual rainfall of 2500 mm – 5500 mm; and maximum and minimum temperature of 35° C and 12° C respectively.

Biochar used in this study was produced from rice husk using a traditional farmer's kiln under oxygen-limited conditions where the organic waste fuel was burnt in the inner cone shaped structure to heat the rice husk in the outer cylindrical drum for over 5 hours, until no visible smoke was emitted from the chimney. The standard biochar properties and total elemental composition produced from rice husk using this method are as given in Table 1 [22].

2.2 Experimental Methodology

The experiment was laid out using a randomized complete block design with three replications. Five treatments were involved which consisted of various combinations of biochar and farm yard manure (FYM). While the quantity of FYM was constant (i.e. 6 t acre⁻¹) in all experimental plots, addition of biochar in the experimental plots differed across treatments as follows: 1. No biochar addition (control; C), 2. Addition of biochar @ 0.8 t acre⁻¹ (B₁), 3. Addition of biochar @1.6 t acre⁻¹ (B₂), 4. Addition of biochar @3.2 t acre⁻¹ (B₃) and 5. Addition of biochar @4.8 t acre⁻¹ (B₄). The chilli variety used in this study was 'sv2319HA' which is a high yielding hybrid variety. The seeds were sown in October in soil potting media in pluck trays under greenhouse conditions and were transplanted in uniform rows 30 days after sowing in a plot size of 6 m² (3m x 2m). Chilli seedlings were spaced 70 cm apart with 70 cm between rows. Uniform intercultural operations were applied to all treatments.

2.3 Data Collection and Statistical Analysis

Composite soil samples were collected before and after biochar addition using random sampling methods from the respective plots. The samples were then analyzed at Soil and Plant Analytical Laboratory (SPAL), National Soil Services Centre, Thimphu to determine the changes in pH, OM, N, Avail P, Avail K, and CEC as affected by biochar addition.

Data on growth parameters - plant height (PH), number of branches (NB), stem diameter (SD) and plant canopy (PC) were recorded at 20 days interval after transplantation upto 40 days. Yield parameters - single pod weight (SPW), number of pods/plant (NP), pod length (PL), pod diameter (PD), Pod weight/plant (PWP), and yield (Y) were estimated from three harvests.

The multivariate analysis of variance (MANOVA) for data on all parameters was carried out at P = .05using R studio team (2021), version 1.4.1103. The packages used for statistical analysis were "dplyr" [23], and "agricolae" [24]. Post hoc analysis was conducted using Tukey's HSD for observations with significant variations among the treatments.

3. RESULTS AND DISCUSSION

3.1 Effect of Biochar Application on Growth and Yield of Chilli

Significant variations were observed among the treatments only in terms of yield, pod weight/plant, number of pods/plant and number of branches/plant (Table 2). However, results also indicated an overall trend of linear increase in fruit yield and plant traits of chilli with the corresponding increase in biochar addition till B₃.

With further increase in biochar rate from B_3 , there was a decrease in yield and plant height, plant canopy, pod weight, number of pods and pod size at B_4 .

The biochar application has significantly influenced the yield of chilli. Although an increase in biochar rates led to a corresponding increase in the yield, the application of 3.2 t acre⁻¹ (B_3) significantly increased the yield (0.95 t acre⁻¹) only over control (0.346 t acre⁻¹). However, the vield of chilli decreased at B₄. Pod weight/plant of chilli was significantly affected across the treatments (P=.03). Similar to yield, B₃ has the highest pod weight/plant (93.99g) and is significantly greater than C - Control (34.2g). The number of pods/plants also differed significantly among the treatments (P = .04). In relation to C (control) significantly higher number of pods were recorded in B₂ (27). The biochar application did not significantly influence plant height, stem diameter, plant canopy, single pod weight and pod size although there was an overall increasing trend till B₃.

The findings of this study align with the review of [25,26] where increased growth of plants due to increased plant photosynthetic rate and enhanced root elongation stimulating plant growth and yield through biochar application was reported. Similarly, an increase in yield of red chilli by 66% for biochar-treated soil was found over the yield in pure acid soil although only minimal difference between biochar treated soil was found suggesting that larger quantities of biochar do not evidently affect crop yield [26]. Biochar application also significantly increased yield, dry matter content and specific gravity of potato tubers [27].

The observed increase in yield and yield components could be due to improved soil physical and chemical properties as reported in various studies: reduced bulk density (-4.5% and -6% with the addition of 2.25 t ha-1 and 4.5 t ha ⁻¹ respectively) [28], increased water holding capacity (25 to 36% increase) with 7% biochar by weight addition [29] and 11% increase [30] liming effect (20 g kg⁻¹ biochar increased soil pH by almost 1 pH unit) [31], and enhanced nutrient availability (significant increases in N (up to 7%), organic C (up to 69%), and P, K, Mg and Ca, [31], increased soluble P and P retention by biochar [32], adsorption of salts [33,34]. The increase in yield and yield components in the experiment suggests that biochar application contributed to the pool of nutrients available in the soil as biochar itself generally contains high

Table 1. Properties and Elemental composition of rice husk biochar

	Wt. Loss (%)	Ash (%)	C (%)	N (%)	рН	Si (mg/kg)	Al (mg/kg)	Ca (mg/kg)	Mg (mg/kg)	Na (mg/ kg)	K (mg/kg)	P (mg/kg)	S (mg/kg)	Fe (mg/ kg)	Mn (mg/ kg)	Zn (mg/ kg)
Rice Husk Biochar	56	44	46	0.63	9.7	193748	212	1340	1683	12353	8502	2983	89	220	454	309

*Wt. Loss: Weight loss

Table 2. Effect of different doses of biochar on yield and yield attributes of Chilli

Т	NB	PH (cm)	SD (cm)	PC (cm)	SPW	NP	PL (cm)	PD (cm)	PWP (g)	Yield (t.acre ⁻¹)
	b				(9)	6			b	
С	23.05	30.70	0.63	25.01	2.99	13.79 [°]	7.69	0.74	34.24 °	0.34 5
B1	23.72 ^b	33.86	0.67	27.81	3.01	24.11 ^{ab}	7.74	0.77	42.94 ^{ab}	0.43 ^{ab}
B ₂	31.55 ^{ab}	42.16	0.84	32.29	3.19	27.14 ^a	7.99	0.76	78.39 ^{ab}	0.79 ^{ab}
B ₃	47.41 ^a	44.56	0.96	39.28	3.36	26.48 ^{ab}	7.66	0.76	93.99 ^a	0.95 ^a
B ₄	33.36 ^{ab}	39.65	0.83	33.70	2.84	22.51 ^{ab}	7.52	0.74	57.20 ^{ab}	0.57 ^{ab}
Pvalue	.03	.07	.07	.052	.10	.04	.76	.71	.03	.03
CV (%)	25.95	14.95	16.89	15.95	6.90	21.03	5.70	3.93	33.40	33.38
SD	11.42	7.34	0.16	6.51	0.25	6.29	0.39	0.02	28.48	0.28

*Means with same letters are not significant at P=.05; T. Treatment, C. Control (No biochar application), B1. Biochar application @ 0.8 t acre-1, B2. Biochar application @ 1.6 t acre-1, B3. Biochar application @ 3.2 t acre-1, B4. Biochar application @ 4.8 t acre-1, NB. Number of branches, PH. Plant height, SD. Stem diameter, PC. Plant canopy, SPW. Single pod weight, NP. Number of pods/plant, PL. Pod length, PD. Pod diameter, PWP. Pod weight/plant, CV. Coefficient of variation, SD. Standard deviation

Table 3. Pearson's correlation for quantitative traits of chilli

	PH (cm)	NB	SD (cm)	PC (cm)	SPW (g)	NP	PL (cm)	PD (cm)	PWP (kg)	
PH (cm)	1.00**									
NB	0.77*	1.00**								
SD	0.92**	0.87**	1.00**							
PC (cm)	0.83**	0.84**	0.92**	1.00**						
SPW (g)	0.31	0.62	0.43	0.37	1.00**					
NP	0.36	0.30	0.38	0.39	0.45	1.00**				
PL (cm)	0.33	0.40	0.38	0.15	0.57	0.15	1.00**			
PD (cm)	0.35	0.47	0.46	0.52	0.50	0.34	0.48	1.00**		
PWP	0.84**	0.84**	0.90**	0.79**	0.61	0.59	0.48	0.46	1.00**	

*Significant at P=.05; **Significant at P=.01; PH. Plant height, NP. Number of pods/plant, NB. Number of branches, PL. Pod length, SD. Stem diameter, PD. Pod diameter, PC. Plant canopy, PWP, Pod weight/plant, SPW: Single pod weight

densities of nutrients [8]. Reported a higher increase of 20% - 120% in crop productivity with biochar addition. Maize grain yield can be significantly increased by 98% - 150% after the application of biochar [35]. [36] Reported 8.8% -14% increase in rice yields. A study also observed that biochar application at 10% (w/w) reduced cumulative leaching of P (37.7%), NH4+ (50.2%), and nitrate (NO –) which could either be due to the enhanced adsorption of these ions to of biochar. the surface the increased immobilization by the greater microbial biomass resulting from biochar addition, or both [37].

In addition, growing crops with biochar resulted in an 11% increase in the average yield on dryland soils [38]. [39] and [25] observed an increase in the OM content of sandy soil by (42-72%) and (18-70%) respectively. In sandy loam, biochar application translated to greater water use efficiency for plants grown under moisture limitations [40].

The chilli plants performed significantly better in the plots amended with biochar when compared to non-amended plots (Table 2). It may be attributed to the long-term beneficial effects of biochar application on microbial growth upon release in soil solution as reported in a review conducted by [25]. Few studies also reported 6.6 to 31.2% higher fungal abundance upon biochar application to the soil which may be due to its suppressive effect pathogens on [41]. Corroborating earlier findings another study stated that biochar application to an asparagus field soil led to a decrease in root lesions caused by Fusarium oxysporum, F. asparagi, and F. proliferatum compared to the non-amended control [42].

The decrease in yield, yield components, and growth in chilli with a higher dose of biochar application in the current study agrees with the findings of [43] and [44] where both reported the negative effect on microbial biomass when biochar was applied at higher concentration. Similarly, [45] also reported the presence of volatile compounds such as benzene in biochar which had detrimental effects on soil microbial biomass. [46] Also noted is that high concentrations of biochar could have a negative impact on soil microbes through the suppression of biological N fixation. Similarly, [26] also reported that larger quantities of biochar do not substantially affect crop growth in red chilli grown in heavy acid soil.

3.1.1 Correlation for quantitative traits of chilli

The correlation analysis for plant traits (PH, NB, SD, PC) and fruit traits (SPW, NP, PL, PD, and PWP) of chilli revealed a high (>0.7) significant (P<.01) positive correlation of PWP (fruit yield) with PH, NB, SD and PC (Table 3). [47] Also reported a significant and positive correlation between fruit yield per plant with fruit weight and the number of branches in chilli. [48] Reported similar results where the number of branches. stem diameter and canopy width had positively significant phenotypic and genotypic correlations with fruit yield. PWP was positively correlated with SPW, NP, PL, and PD although it was nonsignificant (P>.05). [49] also reported a positive association of fruit vield with fruit length and fruit diameter in a similar correlation study carried out in hot pepper.

While PH was significantly and positively correlated with NB, SD, PC, and PWP, NP had a weak positive correlation (<0.2) with PL. [50] also found a positive correlation between PH and SD, and a negative correlation for NP and PL in a similar correlation analysis conducted in chilli. NB had a high positive (>0.7) significant correlation with PC which is plausible since a higher number of branches could result in a larger canopy width. Correlation analysis helps determine the traits on which selection should be based for plant breeding purposes [51]. Hence, as per the results of the study, strong positive correlations of plant traits with fruit yield (PWP) can be considered for indirect selection in breeding programs.

3.2 Effect of Biochar on Soil Chemical Properties

The initial soil condition of the study area had an average pH of 4.3 (strongly acidic) and a sandy loam texture. The soil had very low N, P, and K content with an average value of 0.20% and 23.08 mg/kg and 7.4 mg/kg respectively. Average Cation exchange capacity, soil carbon, and organic matter were also recorded with 16.3me/100g,1.8%, and 3.1% respectively. All the values indicated that the soil had poor fertility and only a small amount of nutrients were available to the plants. We recorded changes in soil chemical properties caused by the application of biochar into the soil. The addition of biochar significantly influenced the soil pH. Changes in avail N, P, K, C, OM, and CEC were also recorded, although no significant differences were recorded statistically (Table 4).

Treatment	pH ^{2°} -pH ^{1°}	$N^{2}-N^{1}$ (%)	P ² -P ¹ (mg/kg)	K ² -K ¹ (mg/kg)	C ² -C ¹ (%)	OM ² -OM ¹ (%)	CEC ² -CEC ¹ (me/100g)
С	0.10 ^a	-0.05	10.99	16.50	-0.10	-0.17	-3.66
B ₁	1.28 ^b	-0.02	27.85	11.53	-0.03	-0.05	11.62
B ₂	0.85 ^b	-0.10	20.43	18.55	0.23	0.40	3.20
B ₃	1.13 [⊳]	-0.07	29.59	11.85	-0.30	-0.51	11.44
B ₄	1.11 ^b	-0.06	8.49	18.84	-0.06	0.11	2.32
P value	.00	0.52	0.51	0.96	0.52	0.52	0.22
C.V.	55.70	0.00	75.10	45.80	113.40	113.40	49.60

Table 4. Between-treatments comparison of mean differences observed in soil properties before and after biochar addition

*Means with the same letters are not significantly different at P=.05; ² Value after biochar addition; ¹ Value before biochar addition C. Control (No biochar application), B1. Biochar application @ 0.8 t acre-1, B2. Biochar application @ 1.6 t acre-1, B3. Biochar application @ 3.2 t acre-1, B4. Biochar application @ 4.9 t acre-1, pH 1. Soil pH before biochar addition, pH 2. Soil pH after biochar addition, N 1. Available nitrogen before biochar addition, N 2. Available nitrogen after biochar addition, C 1. Soil carbon before biochar addition, C 2. Soil carbon after biochar addition, O.M. 1. Soil organic matter before biochar addition, O.M. 2. Soil organic matter after biochar addition, P 1. Available phosphorus before biochar addition, P 2. Available phosphorus after biochar addition, K 1. Available potassium before biochar addition, K 2. Available potassium after biochar addition CEC 1. Cation exchange capacity before biochar addition, CEC 2. Cation exchange capacity after biochar addition

The increase in pH was significantly different between control and treatments (P=.00) with the maximum increase in B1 (1.28). However, progressively increasing the amount of biochar from B1 to B4 did not substantially lead to a corresponding increase in soil pH. Although soil available P, K, and CEC also increased with addition. the increase biochar was not significantly different among the treatments (p>0.05). The maximum increase in P, K, and CEC was observed in B3, B4, and B1 respectively.

Available N decreased with biochar application although the extent of decrease was not significant between the treatments (P>0.05). The maximum and minimum decline were observed in B2 (-0.10mg) and B1 (-0.02 mg). Soil carbon content decreased with the application of rice husk biochar except for B2 (0.23 %). However, the decrease in C was not significant across the treatments (p=0.52). C (-0.17%), B1 (-0.05 %) and B3 (-0.51 %) indicated decline in O.M. content while B2 (0.40 %) and B4 (0.11 %) showed increase in O.M.

Biochar addition induced overall changes in the soil's physical and chemical properties. The increase in pH due to biochar addition was observed in various studies [26,52]. This increase in soil pH may be due to the high pH value of added biochar which can increase soil base saturation. decrease the level of exchangeable aluminum, and consume soil protons [53]. The increase in pH may also be explained by the alkalinity of added biochar which can act as a liming agent in acidic soil [54,55]. Biochar reduces soil acidity by 31.9% through liming effect [56,57,58,59]. Biochar addition alters nutrient cycles by directly adding nutrients (N, P, K, Ca, Mg) in biochar to the soil [60] and by indirectly influencing the exchange of nutrients due to increased reactive surfaces Several mechanisms [61,62]. have heen proposed for the increase of soil nutrient availability including 1) the initial addition of soluble nutrients from biochar [63] and the mineralization of the labile fraction of biochar [64]; 2) reduction of nutrient leaching due to biochar's physicochemical properties [65]; and 3) reduced N losses by ammonia volatilization and N2O from denitrification [66,67].

Biochar application has been found to improve soil phosphorus availability to plants under arid and semiarid agroecosystems since biochar alters soil available P by acting as a P source itself, altering P solubility through changes in soil pH [68] or by promoting P solubilizing bacteria [69]. Biochar has a significant amount of soluble P salts generated during the charring of organic materials [70]. According to [71] biochar's liming impact on acid soil causes Al and Fe to precipitate as Fe(OH)3 and Al(OH)3, increasing phosphorus availability in the soil system; P availability is greatest in the pH range of 5.5-6.0.

The application of biochar increased % organic matter, available K (mg/kg), and CEC which agrees with the study conducted by [26] who reported that biochar application improves acid soil properties (pH, % OM, and % OC) to the levels needed to support plant growth. The possible reason for increased K and CEC might be due to the interaction and reaction of biochar with soil in the short term, such as adsorption and desorption, dissolution, precipitation, and redox reactions [72]. It could also be due to the presence of strong carboxylic and phenolic functional groups on the surface of the biochar particles [73,25] or due to the presence of cation exchange sites on its surface. The increase in K may be due to biochar's porous structure, large surface area, and negative surface charge which can increase the soil's CEC and allow for the retention of nutrients, including K [74].

Biochar has also been shown to increase cation exchange capacity (CEC) by 1.9% [74] which increases the potential for sorption of many organic and inorganic substances, including essential plant nutrients [75,76]. Applying biochar into the soil increases CEC over time due to biochar surface oxidation and the abundance of negatively charged surface functional groups [77,78]. A 2-year field experiment in degraded uplands of East Java, Indonesia (tropical) reported an increase in CEC on biochar application which was attributed to high surface negative charge resulting from oxidation of carboxylic and phenolic groups of biochar [17].

[79] indicated that the biochar treatments significantly increased CEC by 4 to 30% compared to the controls. Similarly, [80] also reported a 21 % increase in CEC in soil following the application of biochar produced from rice husk and sawdust. The Organic matter content of the soil increased with increasing doses of biochar which could be because biochar also undergoes biodegradation, ages, and weather, further increasing CEC [76] although it is considered stable in the soil system [81].



Fig. 1. Values of soil pH, Avail N, Avail P, Avail K, Soil carbon, OM and CEC before and after application of biochar

The current study found a decrease in Available N and the corresponding increase in pH. [82] stated that the decline in soil nitrogen is caused by the increase in soil pH promoting the transformation of ammonium nitrogen into nitrate nitrogen leading to a reduction of available soil nitrogen. Few studies also indicated that the biochar is composed of 0.55 - 1.13 % N suggesting that the biochar application is not increasing the amount of nitrogen available in the soil [83]. The rates tested in this study were closely matched by some of those in the metaanalysis (1.5, 3, 5, 8 Mg ha-1). These rates did not show significant changes due to biochar application which suggests that very small fractions of biochar N are insoluble or readily mineralized forms.

4. CONCLUSION

The subtropical region of Bhutan experiences annual rainfall ranging from 2500-5000 mm making the soil resource very vulnerable to nutrient leaching, erosion losses, and soil degradation. To reduce land degradation and nutrient leaching losses we must adopt new management options to improve soil productivity and ensure sustainable use of land. The application of biochar proved to improve the soil's chemical properties as well as the performance of chili through the findings of this study. The different doses of biochar studied in this experiment favored the improvement of soil chemical properties such as soil pH, P, K, CEC, O.M. and C. The yield of chili also increased in soils treated with biochar with the maximum yield of 0.975 t acre⁻¹ obtained with the use of 3.2 t acre⁻¹ of biochar. To improve the soil properties and production of chili, biochar dose @3.2 t acre for acidic soil of sub-tropical regions is recommended as per the findings of the study. However, further research pertaining to biochar addition in different doses and its impact on crop yields is necessary to further validate the findings.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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