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RESEARCH ARTICLE

# Elemental analysis of humic acid through FTIR and GCMS method and evaluation of humic acid as bio-stimulant for enhancing the yield and quality of rice

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## Abstract

The use of humic substances (HS) as a bio-stimulant emerged as one of the sustainable ways of crop production. The purpose of this study is to analyze the chemical structure of humic acids (HA) through Fourier Transform Infrared Spectroscopy (FTIR) and Gas Chromatography-Mass Spectrometry (GC-MS) methods. This study also aimed to assess the effect of applying HA on the paddy (CO-55 rice variety) to improve the yield and quality of the crop. The experiments were laid out in a completely randomized design with three biological replications in July- 2023 and November-2023-24 at wetland farm (11°0'5.73"N latitude, 76°55'33.16"E longitude for Kharif season and 11°0'7.65"N latitude, 76°55'35.64"E longitude for Rabi season, respectively), Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu. Here, four different concentrations of HA viz. 0.1, 0.2, 0.3 & 0.4% were applied at active tillering and panicle initiation stages of rice along with soil test crop response - integrated plant nutrient system (STCR-IPNS) recommendation-based fertilizers. The application of various treatments showed positive effects in all the parameters of crops viz. growth, yield & quality of rice. Foliar application of HA at 0.3 % recorded the highest grain yield of 5975 and 6245 kg $ha^{-1}$ which was 5.75 and 6.0 % over control treatment and quality of rice grain viz. protein content (11.9 &13.4 %) and starch (93 & 90 %) in Kharif and Rabi season, respectively.

## Keywords

FTIR; GCMS; growth; humic acid; quality; rice; yield

## Introduction

Rice (*Oryza sativa* L.) serves as a fundamental food source for over half of the global population and is cultivated in more than 100 countries, with Asia accounting for 90 % of the world's total production. In 2022, the Food and Agriculture Organization (FAO) of the United Nations estimated that global rice production reached approximately 512.6 million tons. This crop is vital for providing food and energy to more than 50 % of the world's people (1, 2). Rice is nutritionally rich, offering carbohydrates, proteins, fats, vitamins, minerals and a variety of bioactive compounds (3). Food and nutritional security of crops play a vital role in sustaining the health and nutrition of the burgeoning population globally which is expected to increase by 35 % over the next 40 years. However, shrinking cultivable land area coupled with various environmental and soil constraints hinders the targeted yield improvement in many crops to feed the ever-increasing population with the existing farmlands. But in the current situation, climate change is a major element reducing yield and providing a threat to the entire global food production system. Hence identifying best management practices and alternate growth-promoting substances for improving the crop yield is essential.

The application of bio-stimulants in agriculture shows great potential in minimizing the negative effects of climate change, including salt, temperature stress, drought and others. These bio-stimulant products are directly involved in improving nutrient use efficiency, crop yield and quality, reducing anti-nutritional factors and providing stress tolerance but they do not directly add nutrients to the soil (4-8). These products could also be used as additives to fertilizers thus reducing the use of fertilizers.

There are mainly three major groups of bio-stimulants based on their source and content. These groups include HS, hormone-containing products (HCP) and amino acid-containing products (AACP) (9). Humic substances are formed through the decomposition of plants, animals and microbial residues, as well as the metabolic processes of soil microorganisms and makeup around 80 % of soil organic matter (SOM) (10). These substances are categorized into HA, fulvic acids (FA) and humins, depending on their solubility in water or acidic and alkaline solutions (11).

Humic acid plays a key role in enhancing crop growth by stimulating growth-promoting hormones and improving nutrient uptake (12). They act as potent foliar activators, boosting plant growth, root development and yields by enhancing soil's physical and biochemical properties. Humic acids also improve soil fertility by increasing microbial populations, water retention and soil structure (13-15). Additionally, they promote nutrient availability and reduce the transport of harmful heavy metals (16). However, their effects on crops and soil vary, requiring careful application for optimal outcomes. This research investigates the impact of foliar application of HA on rice crops and characterizes the HA using FTIR and GC-MS analysis.

## Materials and Methods

### Characterization of HA

Characterization by elemental composition through FTIR analysis: For the identification and characterization of unknown compounds from HA, FTIR analysis was done. The elemental composition was determined using the FT/IR-6800 type A instrument model. FTIR analysis was performed with a standard light source and TGS detector. Spectra were acquired at 4 $cm^{-1}$ resolution and the scanning speed was 2 mm $sec^{-1}$.

Characterization by elemental composition through GCMS analysis: GC-MS can be used to separate complex mixtures, measure the concentration of analytes, identify unknown compounds and detect trace amounts of contaminants. GCMS analysis was carried out on a Perkin Elmer Clarus SQ8C instrument and gas chromatograph interfaced to mass spectrometer instrument employed the following condition: column: DB-5 MS capillary standard nonpolar column. The sample injection quantity was 1 µl, the dimension was 30Mts, ID: 0.25 mm, film: 0.25 IM and the carrier gas was helium (He). For GCMS, 50 ml liquid HA and 50 ml ethyl acetate were mixed in a separating funnel and collected in two different layers (upper and down portion) separately. Kept this sample for evaporation at room temperature until the solution dried. After the solution was dried, 2 ml of methanol HPLC grade was added in this beaker to dissolve and 2 ml of the sample was collected in a centrifuge tube and processed for GC -MS analysis.

### Experimental Site and Design

A field experiment was conducted to study the efficacy of HA on the growth, yield and quality of rice. The experiments were laid out in a randomized block design with nine treatments and three biological replications in CO 55 rice variety during Kharif-2023 and Rabi 2023-24 at the wetland farm (11°0'5.73"N latitude, 76°55'33.16"E longitude for Kharif season and 11°0'7.65"N latitude, 76°55'35.64"E longitude for Rabi season, respectively), Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu.

### Technical programme

Humic Acid was applied as a foliar spray in four different concentrations (0.1, 0.2, 0.3 & 0.4 %) at active tillering and panicle initiation stages of rice along with STCR-IPNS-based fertilizer recommendation. The treatment details are as follows in Table 1.

**Table 1.** Treatment details

| Treatment Code | Treatment Details |
|---|---|
| $T_1$ | STCR-IPNS-based fertilizer recommendation |
| $T_2$ | $T_1$+ 0.10% HA foliar spray once at active tillering stage $ha^{-1}$ |
| $T_3$ | $T_1$+ 0.10% HA foliar spray twice at active tillering and Panicle initiation stages $ha^{-1}$ |
| $T_4$ | $T_1$+ 0.20% HA foliar spray once at active tillering stage $ha^{-1}$ |
| $T_5$ | $T_1$+ 0.20% HA foliar spray twice at active tillering and Panicle initiation stages $ha^{-1}$ |
| $T_6$ | $T_1$+ 0.30% HA foliar spray once at active tillering stage $ha^{-1}$ |
| $T_7$ | $T_1$+ 0.30% HA foliar spray twice at active tillering and Panicle initiation stages $ha^{-1}$ |
| $T_8$ | $T_1$+ 0.40% HA foliar spray once at active tillering stage $ha^{-1}$ |
| $T_9$ | $T_1$+ 0.40% HA foliar spray twice at active tillering and Panicle initiation stages $ha^{-1}$ |

### Observation analysis

All biometric observations were recorded at different growing stages of plants. Plant samples were collected at active tillering, panicle initiation, flowering and harvesting stages. After the application of HA, all plant growth parameters viz., plant height, root length, leaf area index, chlorophyll content and yield attributes viz., number of productive tillers, grain yield and straw yield were recorded.

### Experimental Soil

The experimental field soil was clay loam in texture with an alkaline pH (8.36), 0.6 EC ($dSm^{-1}$) and medium organic carbon (0.57 %). It has low available nitrogen (219 kg $ha^{-1}$), high available phosphorus (24 kg $ha^{1}$) and potassium (379 kg $ha^{1}$) status.

### Effect of HA on the quality of rice

In the quality assessment of rice grains, the protein content was analyzed using the Kjeldahl method and the starch content was measured using the anthrone reagent technique (17).

### Data analysis

Data analysis from the research is presented in tables and graphs. The results of growth, yield and quality of rice after treatment were correlated to the procedure. Analysis of variance (ANOVA) was performed using General R-based Analysis Platform Empowered by Statistics (GRAPES) software. Means of values were tested by Least Significance Different (LSD) at $P \leq 0.05$ to determine the effects on the efficacy of HA for enhancing the growth, yield and quality of rice.

## Results and Discussion

### Characterization by elemental composition through FTIR analysis

The analysis of the FTIR spectra of HA is shown Fig. 1. The bands value recorded in numerous studies (18, 19). In this graph horizontal axis shows Wavenumber [$cm^{-1}$] and the vertical axis shows transmittance %. In this figure starting and end point 349.053 $cm^{-1}$ and 4000.6 $cm^{-1}$, respectively. According to Libretexts chemistry infrared spectroscopy absorption table, A wide band in the 3600-3200 $cm^{-1}$ range is associated with hydrogen-bonded OH groups found in alcohols, phenols and organic acids, as well as hydrogen-bonded N-H groups. Additionally, a peak between 3000-2500 $cm^{-1}$ corresponds to the C-H stretching vibrations of alkyl, alkene and aldehyde structures. A broad band at 2400-2000 $cm^{-1}$ from O=C=O stretching carbon dioxide, N=C=O from stretching isocyanate, S -C☰N stretching thiocyanate, N=C=N stretching carbodiimide and N=C=S stretching isothiocyanate groups. 2000-1650 $cm^{-1}$ from C-H bending aromatic compound. A band at 1670-1600 $cm^{-1}$ corresponds to the C=C group of alkenes, unsaturated ketone and N-H bending amine group. In 1600-1300 $cm^{-1}$band N-O stretching nitro compound and C-H bending aldehyde group, the second 1400-1000 $cm^{-1}$ from O-H bending carboxylic acid, phenols, S=O stretching sulfate, C-N stretching amine, aromatic amine and CO-O-CO stretching anhydride group. A band at 1000-650 $cm^{-1}$ from C- Cl, C-Br and C-I stretching halo compound and a band at 900-700 $cm^{-1}$ from C-H bending benzene derivatives.

In Fig. 1, all spectra exhibited strong intensity for three bands: 3281, 2259 and 1386 $cm^{-1}$. The first band is attributed to hydrogen-bonded OH groups and/or N-H groups; the second band primarily originates from carbon dioxide and isocyanate compounds; and the third band is mainly due to phenol linkages and O-H groups. In the graph first broad band formed at 3725-3231 $cm^{-1}$ means the presence of Hydrogen bonded OH groups of alcohols, phenols and organic acids, as well as H bonded N-H group, a second broad band formed at the range of 2716 - 2266 $cm^{-1}$ from C-H stretching of aldehyde and isocyanate groups. The appearance of new peaks around 2259-1674 $cm^{-1}$ is attributed mainly to esters, aldehyde and aliphatic ketone-like compound structures. The small band 1386 - 1152 $cm^{-1}$ is attributed mainly to alkane, phenol and esters-like structures (LibreTexts™).

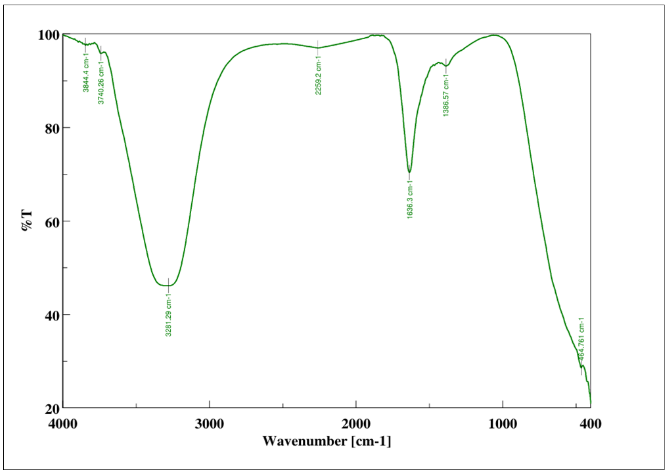


**Fig. 1.** FTIR spectra of HA.

### Characterization by elemental composition through GCMS analysis

Gas chromatography and mass spectrometry were applied to determine the structure of HA. To separate and identify the HA, Perkin Elmer Clarus SQ8C instrument GC-MS was used. 40 different structures were found in the GC-MS analysis of HA is ester, Acetate, nitrile, acids, phenols, amines, aldehydes and different derivative compounds Fig. 2 (A and B). All compounds play an essential role in plant growth and maintaining the sustainability of soil health.

### Effect of HA on Rice

Application of HA had a significant impact on crop yields as well as plant growth parameters in rice (*Oryza sativa* L.) CO-55. This experiment was conducted Kharif 2023 and Rabi 2023-24.

Effect of HA on growth parameters: The result of plant height showed that it varied from 68.3 to 72.0 cm, 86.0 to 94.3 cm and 95.5 to 101.5 cm in Kharif and 73.8 to 83.6 cm, 85.9 to 93.9 cm and 93.9 to 103.4 cm in Rabi at 30 days after treatment (DAT), 60 DAT and 90 DAT, respectively. Among all treatments, $T_7$ recorded significantly higher plant height (72.0 cm, 94.3 cm and 101.5 cm) and (83.6 cm, 93.9 cm and 103.4 cm) at 30 DAT, 60 DAT and 90 DAT in Kharif and Rabi season, respectively, which received 0.30 % HA foliar spray twice at active tillering and panicle initiation stages and lowest plant height was recorded by control treatment (Table 2).

Root length varied from 14.9 to 19 cm and 19.8 to 23.4 in Kharif and from 14.6 to 20.9 cm and 19.6 to 25.8 cm in Rabi season at 30 DAT and 60 DAT, respectively. Among all concentrations 0.30 % foliar spray twice at active tillering and panicle initiation stages recorded significantly highest root length (Table 3). Bio-stimulant treatments significantly influenced shoot and root growth by altering shoot and root structure and development (20) . Humic acids boost root and shoot growth by increasing the production of plant growth-promoting hormones like auxins and cytokinins, as well as enhancing the activity of metabolic enzymes (21, 22). Humic acids applied to rice, results its improved plant height, plant roots, chlorophyll content and yield as compared to the control (23).

Chlorophyll content [SPAD (Soil Plant Analysis Development) Value] increases after the application of HA and the highest value was recorded in $T_7$ (39.8 and 38.2 at 30 DAT) and (44.9 and 47.6 at 60 DAT) in Kharif and Rabi season,

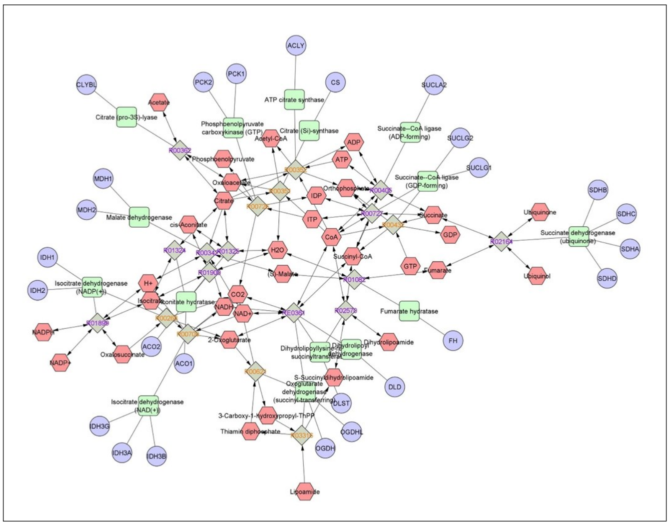

Fig. 2(A). GCMS spectra of HA.

**Table 2.** Effect of HA on plant height of rice

| Treatments | 30 DAT | | 60 DAT | | 90 DAT | |
|---|---|---|---|---|---|---|
| | Kharif | Rabi | Kharif | Rabi | Kharif | Rabi |
| $T_1$ | 68.3 | 73.8 | 86.0 | 85.9 | 95.5 | 93.9 |
| $T_2$ | 64.6 | 76.5 | 86.9 | 87.3 | 95.9 | 95.3 |
| $T_3$ | 67.8 | 78.4 | 88.6 | 89.5 | 98.4 | 97.4 |
| $T_4$ | 70.4 | 78.8 | 88.9 | 90.8 | 99.2 | 98.1 |
| $T_5$ | 68.9 | 80.2 | 91.3 | 91.6 | 99.8 | 98.9 |
| $T_6$ | 70.3 | 82.2 | 92.7 | 93.2 | 100.8 | 101.3 |
| $T_7$ | 72.0 | 83.6 | 94.3 | 93.9 | 101.5 | 103.4 |
| $T_8$ | 70.4 | 80.9 | 91.5 | 92.2 | 98.5 | 99.8 |
| $T_9$ | 69.8 | 81.0 | 91.7 | 92.6 | 100.2 | 100.6 |
| **SEd** | **0.60** | **0.62** | **1.91** | **1.35** | **3.94** | **3.86** |
| **CD ($P \leq 0.05$)** | **NS** | **NS** | **4.04** | **4.08** | **7.65** | **7.52** |

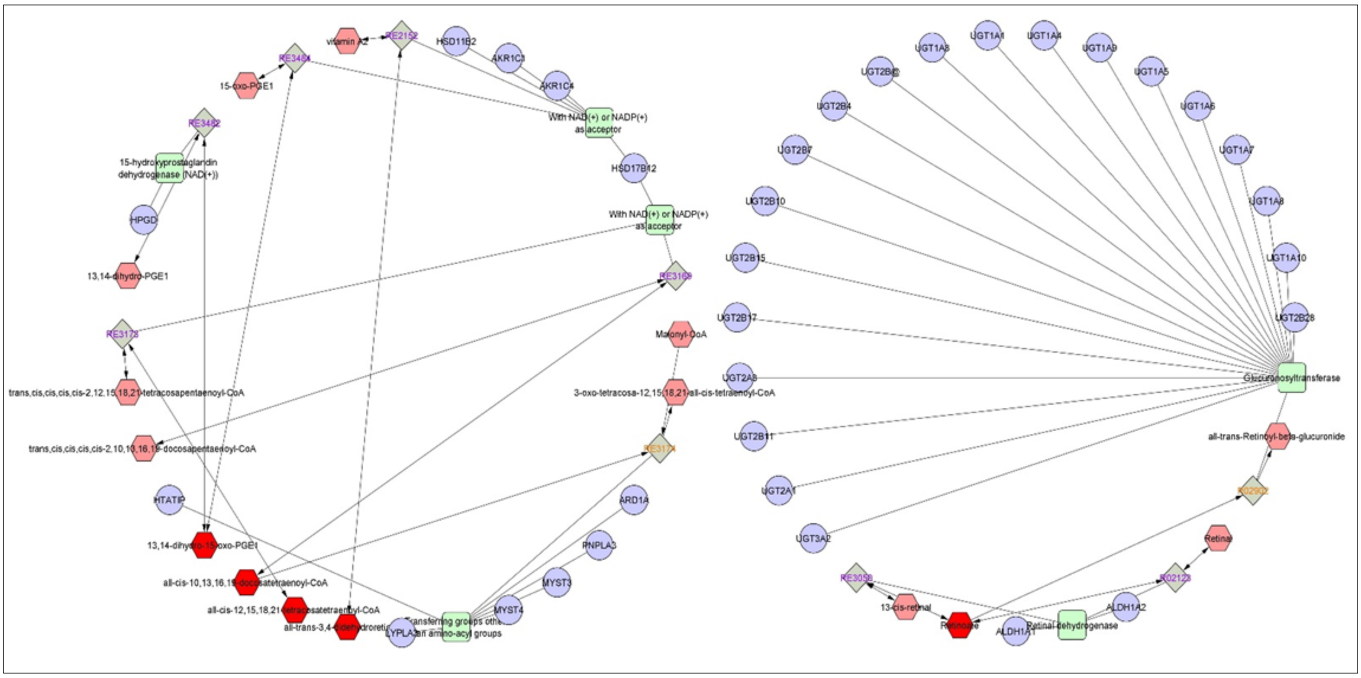

**Fig. 2 (B)**. GCMS spectra with all compounds of HA.

respectively, which received 0.30 % HA foliar spray twice at active tillering and panicle initiation stages and least value recorded control treatment (Table 3). In the leaf area index, at 30 DAT and 60 DAT of rice varied from 1.57 to 2.78 ($m^2$ / $m^2$) and 1.8 to 2.68 ($m^2$ / $m^2$) in Kharif season and 2.08 to 2.82 ($m^2$ / $m^2$) and 2.18 to 2.94 ($m^2$ / $m^2$) in Rabi season, respectively. The largest leaf area index was observed (2.78 and 2.82 $m^2$ / $m^2$) at 30 DAT and (2.86 and 2.94 $m^2$ / $m^2$) at 60 DAT in Kharif and Rabi season, respectively, when a 0.30 % HA foliar spray was applied twice during the active tillering and panicle initiation stages and the control treatment, recorded the lowest leaf area index. Application of HA to sunflower plant grown in Cd stressed soil, result observed that increased growth and chlorophyll content in plants (24).

**Table 3.** Effect of HA on root length and chlorophyll content of rice

| Treatments | Root length of rice (cm) | | | | Chlorophyll content (SPAD Value) of rice | | | |
|---|---|---|---|---|---|---|---|---|
| | 30 DAT | | 60 DAT | | 30 DAT | | 60 DAT | |
| | Kharif | Rabi | Kharif | Rabi | Kharif | Rabi | Kharif | Rabi |
| $T_1$ | 14.9 | 14.6 | 19.8 | 19.6 | 34.2 | 32.1 | 38.3 | 37.3 |
| $T_2$ | 15.2 | 15.2 | 20.9 | 20.1 | 35.3 | 33.2 | 38.9 | 38.5 |
| $T_3$ | 16.1 | 17.3 | 21.2 | 21.6 | 36.3 | 34.1 | 39.8 | 40.7 |
| $T_4$ | 16.9 | 18.1 | 21.5 | 22.0 | 36.7 | 35.4 | 41.0 | 41.2 |
| $T_5$ | 17.2 | 18.9 | 22.4 | 23.4 | 38.0 | 35.8 | 41.2 | 41.6 |
| $T_6$ | 18.5 | 20.3 | 22.8 | 25.1 | 39.1 | 37.0 | 42.0 | 45.7 |
| $T_7$ | 19.0 | 20.9 | 23.4 | 25.8 | 39.8 | 38.2 | 44.9 | 47.6 |
| $T_8$ | 17.6 | 19.1 | 21.9 | 23.8 | 38.2 | 36.2 | 41.5 | 42.6 |
| $T_9$ | 18.1 | 19.7 | 22.9 | 24.2 | 38.4 | 36.8 | 41.9 | 44.3 |
| **SEd** | **0.45** | **0.50** | **0.81** | **0.75** | **0.15** | **0.32** | **0.74** | **0.79** |
| **CD ($P \leq 0.05$)** | **1.05** | **1.08** | **1.72** | **1.52** | **0.33** | **0.68** | **1.58** | **1.71** |

Effect of HA on yield and yield attributes: Humic acids significantly improved the yield attributes and yield of rice. The number of productive tillers varied from 10 to 13.4 and 15.6 to 19.3 in the Kharif season and 11.5 to 17.3 and 17.7 to 22.3 in the Rabi season at 60 DAT and 90 DAT, respectively. Among all treatment applications of HA at 0.30 % foliar spray twice at active tillering and panicle initiation stages recorded the highest number of productive tillers (13.4 and 17.3) at 60 DAT and (19.3 and 22.3) at 90 DAT in both Kharif and Rabi season, respectively. The lowest number of productive tillers recorded by control treatment. All these yield attributes contributed positively toward the grain yield and straw yield. Application of 0.30% HA through foliar spray twice at active tillering and panicle initiation stages recorded the highest ($T_7$) grain yield (5975 kg ha$^{-1}$ and 6245 kg ha$^{-1}$) and straw yield (7370 kg ha$^{-1}$ and 7494 kg ha$^{-1}$) in Kharif and Rabi season, respectively (Table 4).

Different studies have shown that bio-stimulants enriched with humic significantly boost the yield of various crops. Specifically, these bio-stimulants have been effective in enhancing the production of rice (23, 25), okra (*Abelmoschus esculentus*) (26), chickpea (*Cicer arietinum* L.) (27), peas (*Pisum sativum*) (28), wheat (*Triticum aestivum* L.) (29) and hot peppers (30). The results are in line with Shukry (31), Ibraheem (32) which proved, that HA improves the growth and yield of rice in salt-affected lands. Here application of HA at 0.3% as foliar spray twice at active tillering and panicle initiation stage of the rice crop recorded the highest grain yield of 5975 and 6245 kg ha$^{-1}$ which was 5.75 and 6.0 % over STCR-IPNS based fertilizer recommendation (control) in Kharif and Rabi seasons, respectively.

**Table 4.** Effect of HA on grain and straw yield of rice

| Treatments | Grain Yield (kg ha$^{-1}$) | | Straw Yield (kg ha$^{-1}$) | |
|---|---|---|---|---|
| | **Kharif** | **Rabi** | **Kharif** | **Rabi** |
| $T_1$ | 5650 | 5886 | 6708 | 7063 |
| $T_2$ | 5672 | 5930 | 6780 | 7116 |
| $T_3$ | 5712 | 6060 | 6814 | 7272 |
| $T_4$ | 5792 | 6095 | 6905 | 7314 |
| $T_5$ | 5845 | 6150 | 7105 | 7380 |
| $T_6$ | 5910 | 6190 | 7292 | 7428 |
| $T_7$ | 5975 | 6245 | 7370 | 7494 |
| $T_8$ | 5840 | 5965 | 7008 | 7158 |
| $T_9$ | 5935 | 6049 | 7122 | 7259 |
| **SEd** | **28** | **22** | **35** | **24** |
| **CD(P ≤ 0.05)** | **56** | **45** | **70** | **52** |

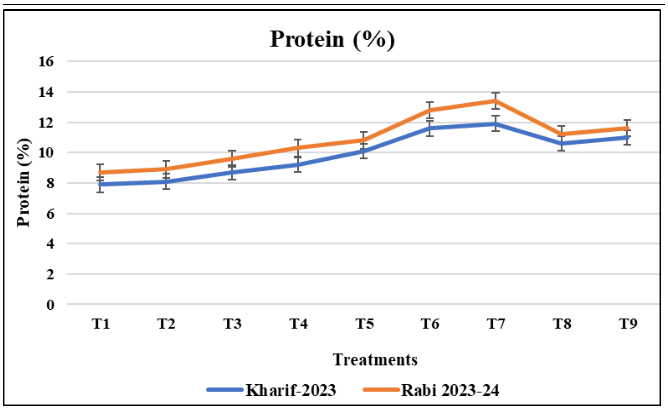


**Fig. 3(A)**. Effect of HA on the protein % of rice grains.

### *Effect of HA on the quality of rice*

There is a little difference observed in protein and starch contents between all treatments. After conducting the statistical analysis, including the t-test, the results indicated that all treatment values were statistically significant Fig.3 (A and B). Among all treatments, application of HA at 0.3 % as foliar spray twice at active tillering and panicle initiation stage recorded the highest protein (11.9 &13.4 %) and starch (93 & 90 %) content in rice grain. The lowest protein and starch content was recorded in the control treatment in both Kharif and Rabi seasons, respectively. Several studies have reported a positive relationship between the effect of HA on plant growth, yield and quality (33, 34). From a quality perspective, the combination of potassium humate and ammonium nitrate, significantly improved the nutritional profile of hot peppers by increasing their carbohydrate, anthocyanin, ascorbic acid, total polyphenol and tannin content (30). HA is not only beneficial to agricultural crops but also to horticultural and floricultural crops. In field trials, the foliar application of HA has also shown positive effects on gladiolus plants of the 'White Prosperity' cultivar, resulting in longer spikes and rachises, a higher number of florets per spike and greater corm weight, diameter and number of cormels (35). In terms of yield attributes, foliar applications of HA have been shown to enhance flower and fruit setting in cucumbers (*Cucumis sativus*) under field conditions (36). Additionally, when HA is applied as a soil treatment, it boosts fruit set and decreases the incidence of fruit drop before harvest in apples (37). Under stress conditions HA enhances the stress-tolerant capacity of plants. It's improved growth, yield, vitamin C and total soluble solid concentration in tomato crops under heat stress conditions (38) and improved growth and quality of maize in drought. It's improved the drought tolerance capacity of plants (39).

## Conclusion

The characterization of HA was performed using FTIR and GC-MS methods. The results indicated that HA contains an abundance of aromatic and aliphatic structures, including esters, acetates, nitriles, acids, phenols, amines, aldehydes and various derivative compounds. These compounds are directly involved in the metabolic activities of plants and contribute to improved growth and yield. In this study, the application of HA on rice at four different concentrations showed that a 0.30 % HA foliar spray, applied twice at the active tillering and panicle initiation stages, enhanced plant growth parameters such as

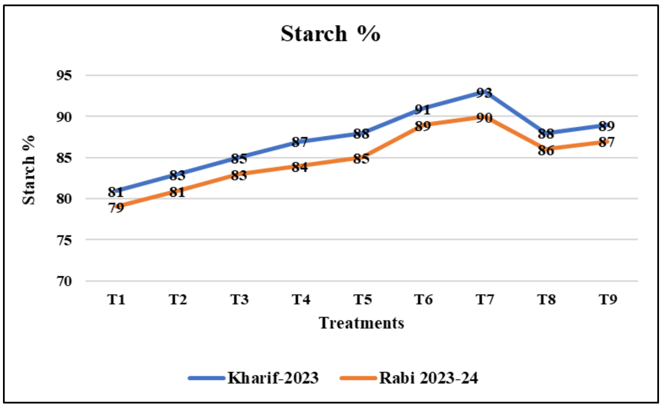


**Fig. 3(B)**. Effect of HA on the starch content of rice grains.

plant height, root length, leaf area index, chlorophyll content and yield attributes, including the number of productive tillers, grain yield and straw yield. The grain yield increased by 5.75 % and 6.0 % over the control in the Kharif and Rabi seasons, respectively.

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## Authors' contributions

The conceptualization of the study was carried out by SRK and SS, while the methodology was developed by the same team. Resources were provided by PB, ST, MG and KDK. Data collection efforts were handled by KSR and KDK and the investigation was conducted by KSR, SS and MG. Formal analysis was performed by KSR, SS and ST. The original draft of the manuscript was written by KSR and SS, with visualization contributions from KSR and PB. Supervision of the study was undertaken by SS, PB, ST, MG and KDK. All authors reviewed and approved the final version of the paper.

## Compliance with ethical standards

**Conflict of interest:** The authors declare no conflicts of interest.

**Ethical issues:** None

### Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript the authors used ChatGPT to correct grammatical mistakes and language editing. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

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