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Application of humic acid and Sulphur on the vigour and viability of harvested soybean seeds with active compounds

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Abstract
Humus represents the outcome of organic matter decomposition facilitated by microorganisms. Humic acid compounds consist of constituents of humus that are extensively distributed across the Earth's surface. Sulphur is a crucial macronutrient in growth and developmental processes, swiftly recognized as the fourth primary plant nutrient, following N, P, and K. The research presented aimed to assess the impact of humic acid and foliar sulphur levels on the vigour and viability of harvested soybean seeds at the Agronomy Research Laboratory, The University of Agriculture Peshawar, during the Rabi season of 2022-2023. A randomized complete block design was chosen in three replications for the conduction of the experiment, which comprises two factors: humic acid levels (0, 3, 6, and 9 kg ha^1) and foliar sulphur levels (0, 4, 6, and 8 kg ha^1), including a control. The analysis of variance demonstrated significant differences among all parameters and their interactions. According to the experimental findings, the application of humic acid at a rate of 9 kg ha^1 resulted in a notable increase in germination (57.67%), seedling fresh (0.89 g) and dry weight (0.69 g), followed by positive yield responses under foliar application of sulphur. Results display the interaction of humic acid levels and foliar sulphur on bioactive compounds, exhibited potential activities in soybean seeds, including isoflavones (12 mg/100 g), saponins (16 mg/100 g), phytosterols (18 mg/100 g), as well as phytic acid (19 mg/100 g) and lectins (22 mg/100 g) in treated as compared to control. In conclusion, applying humic acid at a rate of 9 kg ha^1, combined with foliar sulphur at a rate of 8, is recommended to enhance the vigour and viability of harvested soybean seeds.

Keywords: humic acid, foliar sulphur, vigour and viability of harvested soybean seeds

Highlight:
- Humic acid and foliar sulphur improved the vigour and viability of harvested soybean seeds.
- Humic acid and foliar sulphur enhanced the potential activities of soybean seeds.
- Humic acid and foliar sulphur improved soybean seeds' germination percentage and seedling growth.

1.0. Introduction:
Soybean (Glycine max L.), a legume family member, has gained global significance owing to its adaptability across diverse geographical regions, biochemical composition, and nutritional value (Głowacka et al., 2023; Chris et al., 2005; Chaubey et al., 2000). It is a valuable resource for promoting good health and contributing to diverse and nutritious food options (Delfine et al., 2005; David et al., 2002). Stability and robust performance characterize soybean cultivation, demonstrating resilience even in challenging environmental conditions or water scarcity. It proves effective in crop rotations and with successive plantings (Delfine et al., 2005; El-Ghamry et al., 2009). The N-fixing ability of soybeans depends on various factors such as climate, crop type, and variety. This process enhances soil fertility by converting 50 to 300 kg of ammonia per hectare, depending on soil conditions (Singh, 2017; Ghazal et al., 2013). Soybean oil, a product of soybeans, is rich in essential nutrients. It contains approximately 40% high-quality protein with essential amino acids. Additionally, soybean oil is a source of beneficial antioxidants and omega-3 fatty acids. Its composition includes 23% carbohydrates, 20-24% fat (varying with the soybean variety), and substantial amounts of minerals, vitamins, and fiber (Fukuyama, 2004; El-Shafey & Zen El-Dein., 2016; Puglisi et al., 2009).

Humus is a dark, organic substance that develops in soil and is decomposed by bacteria or through plant and animal matter decomposition. Humic compounds are humic substances extensively distributed on the Earth's surface (Mirnawati et al., 2010). There are three primary types of humic compounds: humic acid (HA), fulvic acid, and humin. Humic acid, in particular, is a valuable substance characterized by numerous functional groups along its chain (Moraes and Rezende, 2008). Humic acid comprises approximately 51% to 57% carbon (C), 4% to 6% nitrogen (N), 0.2% to 1% phosphorus (P), and trace amounts of other elements. It serves as a crucial element within the soil's carbon-based (organic) composition. Scientists and farmers widely utilize humic acid to enhance soil quality and promote plant growth; it is recognized for its positive impacts on enzyme activities, nutrition, and plant growth stimulation and is considered a valuable "plant food" (Getachew et al., 2017; Fagbenro et al., 1993).

Fertilizers amended humic acid enhances photosynthetic efficiency by positively influencing soil hydro-physical properties and nutrient availability (Rady et al., 2016). Studies indicate that the application of 1 kg of humic acid can effectively

replace 1 ton of organic fertilizer (Nardi et al., 2002; Hřivna et al., 2015). Humic acid plays a vital role in supporting plant growth and root development. In legumes, root aperture and dry mass increase with stimulated root growth (Sharif et al., 2002; Eyheraguibel & Morard. 2008). It is also renowned for mitigating soil diseases and enhancing various aspects of soil health, plant nutrition, mineral availability, and fruit quality (Adhikary et al., 2007; Singh & Mena, 2004; Sarir et al., 2005). Bean plants require the provision of magnesium (Mg) for robust development (Rezende et al., 2009; Imran et al., 2021).
Sulphur concentration in plants typically ranges from 1.0 to 3.0 g/kg. Furthermore, sulphur plays a crucial role in fatty acid synthesis, elevates protein quality by facilitating amino acid production, and holds a unique function in the synthesis of enzymes, vitamins, lipids, and other essential plant components (Havlin et al., 1999; Horowitz & Meurer, 2006). The substantial protein content in soybeans is intricately tied to their nutritional value, attributed to the elevated Sulphur (S) levels. In legumes, sulphur fertilization significantly influences various storage proteins, including albumin, globulin, prolamin, and glutenin (Chandra & Pandey, 2016; Bakry et al.,2015).
The current search aims to evaluate the effects of humic acid and foliar sulphur spray in legumes (soya bean plants) and investigate their impact on growth rate and bioactive compounds with quality and yield.

Materials and Methods:

The experiment was conducted in the Agronomy Research Laboratory at the University of Agriculture Peshawar during Rabi season 2022-2023. The experiment was designed in a randomized, complete block design with three replications. Materials used in this research were three replications of soybean seeds grown under two factors, where one factor was Sulphur levels (0, 4, 6, 8 percentage points), and the other was humic acid levels (0, 3, 6, and 9 kg ha^{-1}) with control(Table 1). The harvested soya bean was subjected to tests for vigour and viability.

Table:1 Application factors

Factor A: Humic Acid (kg ha^{-1})	Factor B: Foliar Sulphur (%)
HA_1 = 0 HA_2 = 3 HA_3 = 6 HA_4 = 9	S_1 = 0 S_2 = 4 S_3 = 6 S_4 = 8

Parameters studied

1.1.1. Standard germination test

A standard germination test was conducted for seeds described in the ISTA (1999). Four replicates of 50 seeds from each line were tested. Wet filter paper was used as the germination medium for soybean seeds. The number of normal seedlings was estimated after a 7-day incubation period at 25°C.

1.1.2. Germination percentage (%)

The germination percentage was calculated using the following formula.

$$\text{Germination \%} = \frac{\text{Total sprouted seeds}}{\text{Total seeds}} \times 100$$

1.1.3. Germination rate

The germination rate was calculated by following the formula

$$\text{Germination rate} = \frac{\text{Total number of seeds sprouted}}{\text{Total days for germination}}$$

1.1.4. Root, shoot, and seedling Fresh Weight (g):

The five seedlings from each treatment were selected to measure the fresh weight of the shoot, root, and seedling. Total seedling fresh weight was determined as follows;

Total seedling fresh weight = root fresh weight + shoot fresh weight

Dry weight was calculated by putting the root and shoot in an oven at 70°C for 48 hours till constant weight was achieved and noted to determine the dry weight (g).

1.2. HPLC Analysis

High-performance liquid chromatography (HPLC) is a widely used analytical technique for separating and quantifying active compounds that involve several steps. Overall, measuring active compounds in HPLC involves precise sample preparation, chromatographic separation, detection, quantification, and data analysis to determine the concentration of the compounds in the sample accurately. The soybean seeds sample was prepared by extracting the compounds from the matrix and filtering them to remove particulate matter. The prepared sample was injected into the HPLC system, where the active compounds were separated based on their chemical properties, such as size, polarity, and charge. This separation is achieved by passing the sample through a stationary phase (the column) and a mobile phase (the solvent). As the separated compounds elute from the column, they pass through a detector that measures their concentration. The concentration of the active compounds is determined by comparing the peak areas, or heights, of the compounds in the chromatogram to a standard calibration curve. The calibration curve was generated by analyzing standard solutions at known concentrations of the active compounds (Ahmed et al., 2021).

1.3. Statistical analysis:

The data was statistically analyzed using the technique of two sample T-tests at a probability level of 5% using the statistical software Statistix 8.1. Data Analysis: The data obtained from the HPLC analysis is processed using specialized software to calculate the concentrations of the active compounds in the sample

2.0. Results

2.1. Effect of humic acid and foliar Sulphur on germination percentage (%)

The germination percentage of soybeans was evaluated under the influence of varying concentrations of humic acid and sulphur and reported in Table (2). The application of humic acid at a rate of 6 kg ha-1 yielded the highest germination percentage (61.00%), whereas the control plants exhibited the lowest germination rate (56.83%). Additionally, foliar application of sulphur at an 8% concentration demonstrated an improved germination percentage (63.17%) compared to the control treatment (58.17%). The data analysis revealed that the interaction between humic acid and sulphur levels had a non-significant effect on the germination percentage of soybeans. This implies that the combined application of humic acid and sulphur did not result in a statistically significant difference in germination compared to their individual effects.

Table 2. Effect of humic acid and Sulphur levels on soybean germination percentage (%) and rate.

HA (kg ha^{-1})	Foliar Sulphur (%)				Mean
	0	4	6	8	
0	54.00	60.00	53.33	60.00	56.83 a
3	59.33	49.00	61.33	60.00	57.50 a
6	62.00	55.33	57.33	69.33	61.00 a
9	57.33	56.00	54.00	63.33	57.67 a
Mean	58.17 a	55.17 b	56.50ab	63.17 a	
Germination rate	Foliar Sulphur (%)				Mean
HA (kg ha^{-1})	0	4	6	8	
0	3.86	4.29	3.81	4.28	4.06 a
3	4.24	3.52	4.38	4.26	4.11 a
6	4.43	3.95	4.09	4.81	4.32 a
9	4.09	3.99	3.86	4.52	4.12 a
Mean	4.15 a	3.94 a	4.03 a	4.48 a	

LSD value ($P \leq 0.05$) for Humic Acid level (HA) = NS, LSD value ($P \leq 0.05$) for Sulphur (S) = NS, LSD value ($P \leq 0.05$) for Sulphur (S)= NS, Interaction of HA x S= NS, means of the same category followed by different lettering are significantly different.

2.2. Seedling fresh and dry weight of soybean (6g)

The fresh seedling weight of soybeans was calculated under varying levels of humic acid and sulphur (Table 3). It was observed that applying humic acid at a rate of 9 kg ha^{-1} resulted in the highest seedling fresh weight (0.89 g) and dry weight (0.68 g), whereas the control plots exhibited the lowest lower fresh weight (0.88 g) and dry weight (0.61 g). Additionally, foliar Sulphur application at an 8% concentration demonstrated enhanced root, shoot, and fresh seedling weight. Upon analyzing the data, it was determined that the interaction between humic acid and sulphur levels had a non-significant effect on the fresh weight of soybeans. It suggests that the integration of humic acid and sulphur did not result in a statistically significant difference in the root, shoot, and seedling fresh weight compared to their individual effects.

Table 3. Effect of humic acid and Sulphur levels on soybean seedling fresh and dry weight (g).

HA (kg ha^{-1})	Foliar Sulphur (%)				Mean
	0	4	6	8	
0	0.88	0.89	0.89	0.88	0.88 a
3	0.87	0.91	0.87	0.88	0.88a
6	0.89	0.89	0.85	0.88	0.89 a
9	0.89	0.88	0.87	0.90	0.89 a
Mean	0.88 ab	0.89 a	0.87 b	0.89 a	
Dry weight	Foliar Sulphur (%)				Mean
HA (kg ha^{-1})	0	4	6	8	
0	0.61	0.69	0.69	0.67	0.68 a
3	0.67	0.71	0.67	0.68	0.68a
6	0.69	0.69	0.66	0.68	0.68 a

9	0.69	0.68	0.67	0.71	0.69 a
Mean	0.68 ab	0.69 a	0.67 b	0.68 ab	

LSD value ($P \leq 0.05$) for Humic Acid level (HA) = NS, LSD value ($P \leq 0.05$) for Sulphur (S) = NS, LSD value ($P \leq 0.05$) for Sulphur (S)= NS Interaction of HA x S= NS, Means of the same category followed by different lettering are significantly different.

2.3. Effect of Humic acid and Sulphur on Active compounds of soybean seeds

The high-performance liquid chromatography (HPLC) analytical technique was applied to determine the effect of humic acid and sulphur on bioactive compounds. The results indicated that four active compounds were measured in the soybean seeds using HPLC. It was revealed that the quantity of these compounds was higher than that of the control (Table 4). These active compounds were isoflavones (12 mg/100 g), saponins (16 mg/100 g), and phytosterols (18 mg/100g). Additionally, the levels of phytic acid and lectins were determined to be 19 mg/100g and 22 mg/100g, respectively. These compounds offer various health benefits, including cholesterol reduction, cancer prevention, immune system modulation, and mineral absorption enhancement. Nevertheless, it is crucial to recognize that the concentration and bioavailability of these compounds can vary based on processing methods, environmental conditions, and genetic variations in soybeans. Thus, accurate and reliable measurement and quantification of these compounds are essential for a comprehensive understanding of their presence and potential health effects (Fig.1 & 2).

Table 4. Analysis of bioactive compounds of soybean by HPLC under humic acid and sulphur levels

Sulphur levels					
		S(%) 0	**S (%)3**	**S(%) 6**	**S(%) 9**
Humic acid	**Before**	Isoflavones	Saponins	Phytosterols	Phytic acid
HA 0 %	10	12	11	10	12
HA 3 %	11	15	13	12	16
HA 6 %	12	18	15	14	17
HA 9 %	19	22	16	18	19

LSD value ($P \leq 0.05$) for Humic Acid level (HA) = NS, LSD value ($P \leq 0.05$) for Sulphur (S) = NS, LSD value ($P \leq 0.05$) for Sulphur (S)= NS, Interaction of HA x S= NS, Means of the same category followed by different lettering are significantly different.

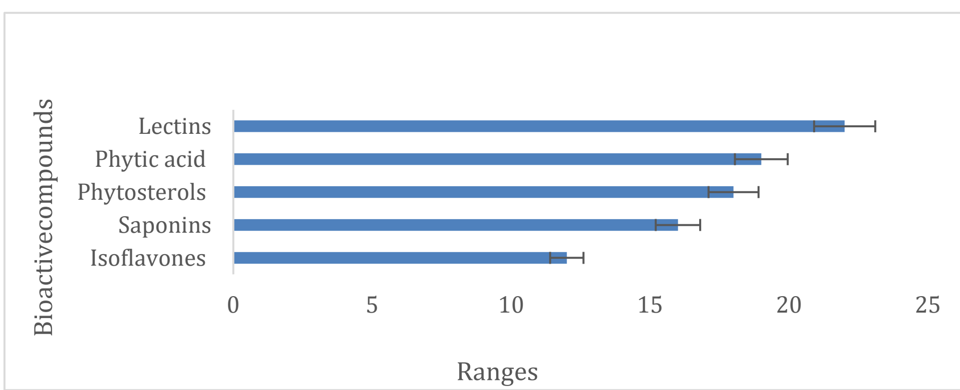

Figure 1: The isolation of active compounds in soybean seeds

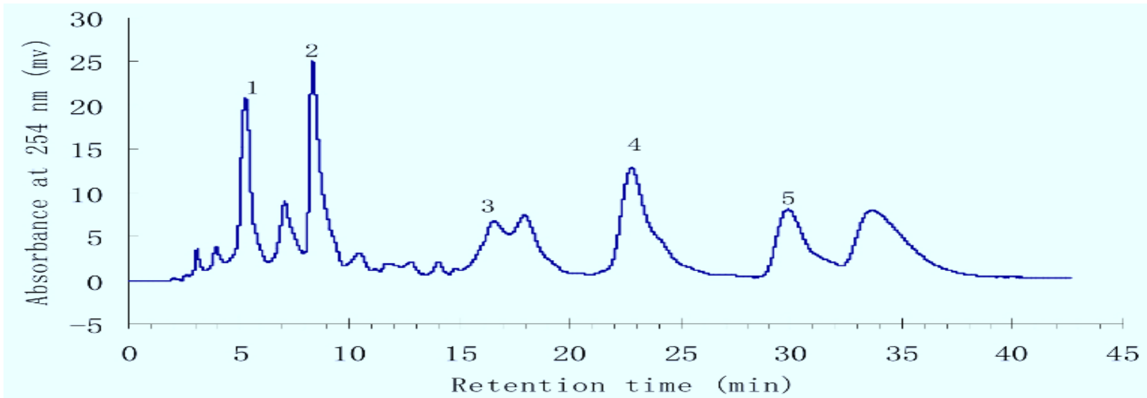

Figure 2: The chromatography of active compounds in soybean seeds with letters 1, isoflavones, 2, saponins, 3, phytosterols, 4 phytic acid, and 5 phytosterols with absorbance at 245 nm after application of humic acid and sulphur

Discussion

Soybean seeds are seeds of a legume plant native to Southeast Asia that contain plenty of protein, fiber and essential nutrients like iron, calcium and vitamin C; they are used to make products such as tofu, milk, sauces, oils, and animal feed and can be eaten whole or as part of many dishes in cuisines worldwide (Azeem et al., 2014; Anjum et al., 2016; Mohamed et al., 2009). Results showed that the germination rate and weight of seedlings were increased under humic acid and sulphur application (Ahmad et al., 1999; Ali & Singh., 2010; Akhtar et al., 2021; Begum et al., 2012; Broch et al., 2010; Burkitbayev et al., 2021). Due to its molecular weight, humic acid is quickly absorbed by the seeds and increases the amount of nitrogen, phosphorus and other products. A detailed literature search showed that results are consistent with those of Biel et al. (2017), who reported that increased foliar Sulphur application increased the fresh root weight of bean plants. Results revealed that fertilization of soybeans with sulphur had a positive effect on various production parameters such as plant height, number of pods per plant, root dry weight, and seed weight per plant. It was observed that humic acid and sulphur content did not significantly affect the germination rate of beans, which was contrary to the reports of Mosa Pour et al. (2014) that humic acid has a positive effect on marigold growth. Ghorbani et al. (2010) found that humic acid at 54 mg/L could increase the germination rate. The soybean seed weight data showed that humic acid and sulphur content did not significantly affect soybean seed weight (Dhaker et al. 2014; El-Bassiony et al. 2010).

The current results are similar to the reports of Sheriff (2002), who reported that the weight of dry beans increases and found 150 mg under humic acid (Tejada & Gonzalez, 2003; Piri et al.,2012). Application of 40 kg S ha−1 significantly increased seedling weight, number of pods per plant, and pod length (Dhaker et al. 2014; Kildisheva et al, 2020). Our results are consistent with those of Biel et al. (2017), who reported that increased foliar sulphur application increased the fresh weight of bean plants. Isoflavones: Isoflavones are a type of phytoestrogen found in soybeans. It was observed that the application of humic acid and sulphur also impacted bioactive compounds, which were found to be more in comparison to the control (Table 4).The health benefits of these compounds include reducing the risk of some cancers, improving bone health, and reducing menopausal symptoms (Noman et al., 2015; Das & Das. 1994). Saponins are natural substances found in many plants, including soybeans. They have been shown to have anti-inflammatory and antioxidant properties.

Additionally, saponins may lower cholesterol and help strengthen the immune system (Saleem et al., 2019). Phytosterols are plant compounds with a structure similar to cholesterol (Nasreen et al., 2006). They are known to help lower LDL cholesterol and reduce the risk of heart disease. Soybeans are a good source of plant sterols. Phytic acid, or phytate, is a compound found in soybeans and other plant foods. It can bind to minerals such as iron, zinc and calcium, reducing their absorption in the body. However, phytic acid also contains antioxidants that may have some health benefits. Lectins are proteins found in many plant foods, including fruit juices. They can be combined with carbohydrates and are associated with many health benefits. Some lectins may have anti-cancer properties, while others may cause digestive problems in some people (Qadoons et al. 2015). These compounds contribute to the nutritional value and health benefits to humans.

Conclusions

It can be concluded that combined fertilization of humic acid at 9 kg/ha and foliar sulphur at 8 kg/ha is recommended as an effective strategy to improve the viability of harvested soybean seeds. The application of current technology may increase the nutrient quality of soybeans as they are a rich source of active compounds that benefit human health. Also, this research increases understanding of plant nutrition and growth that provides practical insights for optimizing soybean seed quality, thereby improving agricultural practices and crop yields.

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