International Journal of Agriculture and Biosciences 2025 14(3): 447-454.
https://doi.org/10.47278/journal.ijab/2025.042

RESEARCH ARTICLE eISSN: 2306-3599; pISSN: 2305-6622

Humic Acid-mediated Resistance in Cotton (*Gossypium hirsutum* L.) against Bollworm (*Helicoverpa armigera*)

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ABSTRACT

Cotton (*Gossypium*) is a globally important fiber crop. Cotton bollworm (*Helicoverpa armigera*) attacks on the boll and interrupted its growth and productivity. Recently, several studies have proven the ability of humic acid (HA), an organic compound obtained from soil humus to improve plant growth and responses against biotic stress. This study evaluates the effects of humic acid on cotton seed germination and callus formation for enhanced bollworm resistance. It is hypothesized that humic acid treatment promotes plant growth, enhances callus induction, and increases resistance to cotton bollworm. The methodology involved the collection of cotton seeds and were surface sterilized. The seeds were germinated on Murashige-and-Skoog's (MS) medium. Grown embryos were transferred to culture tubes and maintained on MS medium with auxin or cytokinin for callus induction. During callus induction and maintenance, humic acid was supplemented in three concentrations: 0.1%, 0.5% or 1%. Growth parameters along with biomass were measured and the efficacy of humic acid on enhancing pest resistance was tested through bioassays against *Helicoverpa armigera* larvae. The findings of the study revealed that humic acid appreciably influenced callus induction. The growth and acclimatization of plants treated with humic acid were significantly higher than untreated control plants. The percentage of death rate on the cotton bollworm in humic acid-treated plants were 60%, compared to untreated plants resulted in the increased resistance against these pests. Hence, this study concluded that humic acid accelerated the growth of cotton, promoted callus induction and increased resistance to cotton bollworm.

Keywords: Cotton, Humic acid, Cotton bollworm (*Helicoverpa armigera*), Insect bioassay, Mortality, MS medium, Callus induction, Seed germination, Integrated pest management

Article History
Article # 25-070
Received: 14-Feb-25
Revised: 10-Mar-25
Accepted: 16-Mar-25
Online First: 23-Mar-25

INTRODUCTION

Cotton is a member of the Malvaceae family, which includes over 200 genera and about 2,300 species in at least ninety-five families. Cotton (*Gossypium* spp.) is the staple of global agribusiness, prized for its hardiness and utility in making textiles, animal feed and countless industrial products. It has worldwide importance as a staple food

Cite this Article as: Ghaffor A, Qadir F, Hafeez A, Chang MS, Khaskheli MA, Khaskheli MA, Suthar V, Kolhi ML, Alam R, Shehzad S, jaweria W, Aslam P, Misbah F, Haleem M, Afzal A, Shoukat A, Rasool G, Zafar MM and Razzaq A, 2025. Humic acid-mediated resistance in cotton (*Gossypium hirsutum* L.) against bollworm (*Helicoverpa armigera*). International Journal of Agriculture and Biosciences 14(3): 447-454. https://doi.org/10.47278/journal.ijab/2025.042

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crop because of its adaptability to contrasting climates, from arid regions and refined contacts growing (Kang and Banga, 2013; Zafar et al., 2023). Cotton holds not just its commercial significance but also it is crucial in food security, rural livelihoods and international trade. The cotton plant exhibits extraordinary adaptability and thrives in a variety of soil and climate conditions. Cotton production includes agricultural practices such as soil management, planting, irrigation, fertilization, pest control, and harvesting. Demand for cotton varieties is high and growers continue to grow crops that are high-yielding, pest resistant and good fiber. Planting methods vary depending on factors such as climate, soil type and growing methods (Shahrajabian & Sun, 2024; Zafar et al., 2024).

Cotton, is generally regarded as the widest-used natural fiber, and represents one of our earliest experiences with fabric. Its properties include absorbency, breathability, and softness, all of which make it suitable for use in clothing. Cottonseeds are used in the production of basketwork as well extraction to produce cotton base oil that is normally employed nationally for cooking. Cottonseed hulls can be used as fuel and in the manufacturing of composite materials, among other industrial applications (Siddiqui et al., 2020; Rekaby et al., 2023). Cotton is affected by bacterial blight, fungal diseases including Alternaria leaf spot and viral infection such as cotton leaf curl virus. Management of these diseases includes growing resistant varieties, soil rotation and proper applications of fungicides / bactericide sprays (Tabashnik and Carriere, 2019; Zafar et al., 2020). The cotton bollworm, *Helicoverpa armigera* is one of the most devastating insect pests to attack worldwide agricultural resources especially crops belonging to order *Lamiales* containing many commercially important plants. It is widely recognized as a key polyphagous lepidopteran pest and causes severe damage to the cotton crop resulting in economic losses worth billions of dollars annually. Therefore, understanding the life cycle and feeding behavior of *H. armigera* and developing effective management strategies against it are extremely important areas of study to minimize its damage (Razzaq et al., 2021). The life cycle of the cotton bollworm (CBW) consists essentially four stages that is egg, larva or caterpillar, pupa and adult form. Adult females lay eggs on the plant, often under leaves or within bolls. Upon hatching, the larvae feed on cotton buds, flowers and bolls. Feeding inflicted by the insects inflict clear, visible damage to aboveground plant parts and can also cause wound sites for secondary infections by pathogens. Larvae develop through a few instars before they pupate in the soil. These adult moths enclose in 1 to 3 days, and are strong fliers with the ability for long-range dispersal that further contributes toward their proliferation and management blunders (Reigada et al., 2016; Zhao et al., 2024).

H. armigera inflicts a huge economic impact with losses in cotton production estimated to be billions of dollars annually. This ability of the pest to evolve into insecticides over time resistance creates a major challenge for effective early management and it needs continuous research towards developing an alternative method. The environmental consequences of the large number of pesticides used are dangerous both to non-target organisms that could be harmed by some agrochemicals and in terms of ecological equilibrium (Chen et al., 2014). Traditional insect control methods, relying primarily on chemical insecticides, have long been the mainstay of cotton disease control. However, this method brings with it many challenges that hinder its effectiveness and sustainability. Pesticides often cause ecological imbalances, including the development of pest populations, negative impacts on non-target species, and environmental degradation (Razzaq et al., 2022). Based on these challenges, there is a growing desire to find other environmental friendly solutions such as humic acid as a way to control pests (Gurr and You, 2016). Environment-friendly pest control methods, also referred to as sustainable organic or green approaches concentrate on the prevention and eradication of pests with reduced environmental impact. So, the need is to get that into a more sustainable method with the assistance of utilizing both biological control (like predator and parasitoids) again as well as cultural practices including crop rotation / proper sanitation for reducing pest habitats. Moreover, mechanical methods such as traps and barriers physically remove or exclude pests with no chemical application. Using resistant crop varieties, obtained by classical breeding or genetic engineering, to mitigate pest harm and cut the use of chemical pesticides. Natural compounds, including neem oil and insecticidal soaps are used in organic pest control as an alternative to synthetic chemicals because they affect non-target species less adversely than would these strong, broad-range chemical materials. The idea behind these practices is to maintain a natural ecosystem balance, promote biodiversity, and decrease environmental stress or health risks associated with traditional means of pest control (Zafar et al., 2021). The aim of this study is to evaluate the impact of humic acid on biomass production in callus and to evaluate the effectiveness of bioassay in treating cotton plants against bollworm disease.

MATERIALS & METHODS

Plant Material Collection

Cotton seeds (*Gossypium* spp.) of high yielding varieties were collected (Fig. 1) from Four Brothers Group Lahore.

Delineating of Cotton Seeds

Cotton seeds were surface sterilized by placing them in a sterile beaker filled with sterile MilliQ ultrapure water and (H_2SO_4) for 1min. The seeds were vigorously shaken 2-3 times with this water to remove any external debris.

Surface Sterilization

The seeds were transferred to a 100mL solution of 70% ethanol mixed with 2 drops of Tween 20, and immersed for 1min to disinfect the surface. Afterward, the seeds were rinsed twice with sterile MilliQ ultrapure water, each rinse lasting for 2mins, to remove residual ethanol. Then, the seeds were treated with a 10% bleach solution, adding 2 drops of Tween 20, and incubated for 1-2mins to ensure thorough sterilization. Finally, the seeds were washed 3-5 times with sterile MilliQ ultrapure water to eliminate any remaining bleach. Finally, the seeds were soaked overnight in sterile MilliQ ultrapure water to soften the seed coat before use in further experiments.

Media Preparation

The Murashige and Skoog (MS) medium, was prepared by dissolving the MS salts, following the manufacturer's instructions, in distilled water. It was prepared by adding 15g of sucrose and added 0.05g of mayo- inositol to a flask and then, flask was filled with distilled water up to a total volume of 500mL and mixed well. The pH of the solution was adjusted to 5.7 using 1N NaOH. For solidification further, 0.8% Phytagel was incorporated into the medium. The prepared medium was sterilized by autoclaving at 121°C for 15-20mins. Once the autoclave reached at 70°C, carefully opened it and transfer the test tubes. 5mL of the prepared medium was poured into each test tube and then the test tubes were covered with aluminum foil.

Seed Germination

Following this, the seeds were germinated in a flask at 30^0C. The embryos were excised and sown on 25mL of Murashige and Skoog (MS) agar medium in a petri dish supplemented with 1% sucrose to serve as germination for 2-3 days. Petri dishes were sealed with Parafilm and transferred to a growth chamber 25°C ±2°C in the presence of 16/8 light/dark period. The embryos were shifted to the culture tubes. The plantlets were grown in the tubes and kept for 2 months (Sarwar et al., 2008).

Induction and Maintenance of Callus Culture (Fig. 1)

The leaves from the plantlets in the tubes were taken for callus formation using the modified Murashige and Skoog (MS) method to control plant growth, which is usually a combination of auxins (such as 2,4-dichlorophenoxyacetic acid [2,4-D] at 1-2mg/L) and cytokinin (such as 6-benzylaminopurine [BAP] at 0.5-1mg/L) to improve callus. The experiment was cultivated in the dark at 25±2°C to promote callus growth, with regular monitoring and culturing every 3-4 weeks on fresh MS medium and the same growth medium to maintain propagation. The quality of the callus culture was evaluated according to its shape, color and distribution, taking into account the severity of the callus and light colors due to its lack of ability to regenerate and respond to treatment (Michel et al., 2008).

Application of Humic Acid (HA)

For humic acid treatment, stock solutions were prepared by dissolving humic acid powder in sterile water to reach 0.1%, 0.5% and 1%, followed by filtration to form sterile solutions. Depending on the application, these results were entered into the MS system when the callus is initiated or during maintenance. Control groups were played an important role in determining the specific effects of humic acid in ensuring key parameters (Rose et al., 2014).

Measurement of Biomass

Biomass parameters are important in evaluating the growth and productivity of callus cultures treated with humic acid. Fresh biomass was determined by measuring callus samples immediately after harvest using a constant safety method, while dry biomass was measured after drying the samples to constant weight in an oven at a specific temperature. The difference between fresh and dry weight has given an idea about the water and normal biomass of the collected callus cultures. Biomass measurements were taken daily over a period of time throughout the research period to determine the power of the explosion and the results of the humic acid on reduction of cotton bollworm (Pereira et al., 2019).

Insect Bioassay

The efficacy of humic acid was tested as insect bioassay against *Helicoverpa armigera* larvae by comparing toxicity of control cotton plant (Fig. 1). The leaves of cotton plant with *Helicoverpa armigera* were taken on a moist filter paper in a petri dish. A 5-7 larvae of 3^{rd} instar were taken and mortality rate was measured (Koul et al., 2023).

% Mortality = Number of dead larvae ⁄ Total Notes × 100

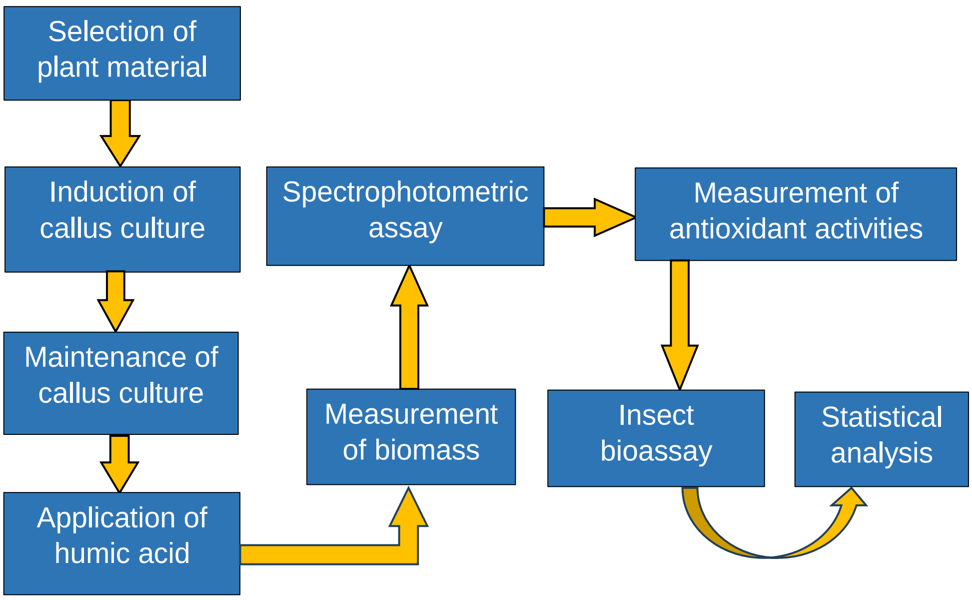

Fig. 1: Schematic representation of overall methodology.

RESULTS

Germination of Cotton Seeds

The cotton seeds over surface sterilization were incubated for germination. The germination of embryos was observed over a period of 24h (Fig. 2).

Fig. 2: Germination of cotton seeds for embryos.

Shifting of Embryos onto the Plates

The embryos were excised from the seeds and shifted to the plates and observed enlargement over a period of 48-72h (Fig. 3).

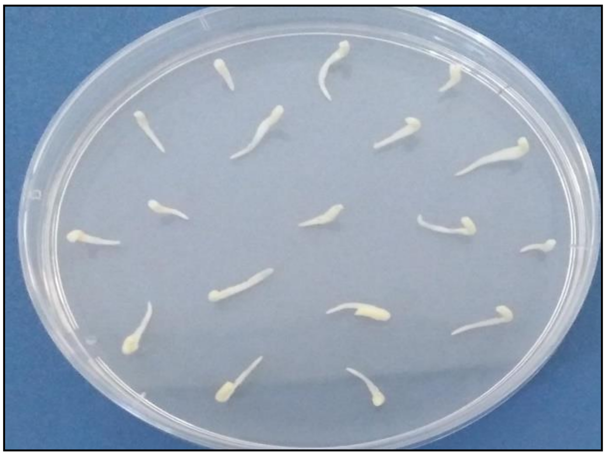

Fig. 3: Placing embryos on plates containing MS medium.

Shifting of Embryos on to the Tubes

The enlarged embryos were then shifted to the culture tubes and placed growth room at 25°C ±2°C in the presence of 16 light; hour/ 8 dark (Fig. 4).

Shooting of Embryos in Tubes

The tubes containing embryos supplemented with growth regulators started shooting over a period of 2 weeks. The emergence of roots and shoots were observed inside the culture tubes (Fig. 5).

Callus Induction

The leaf explant was taken from the plantlet grown inside the tube, and placed on/in the plates and tubes containing callus inducing medium. The formation of callus was observed. The supplementation of humic acid was done and seen at callus formation stage (Fig. 6).

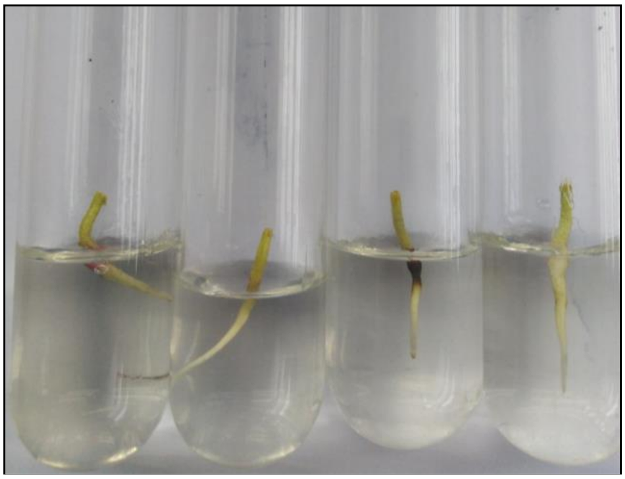

Fig. 4: Shifting embryos into the tubes containing MS medium supplemented with growth regulators.

Fig. 5: Emergence of roots and shoot in tubes containing MS medium supplemented with growth regulators.

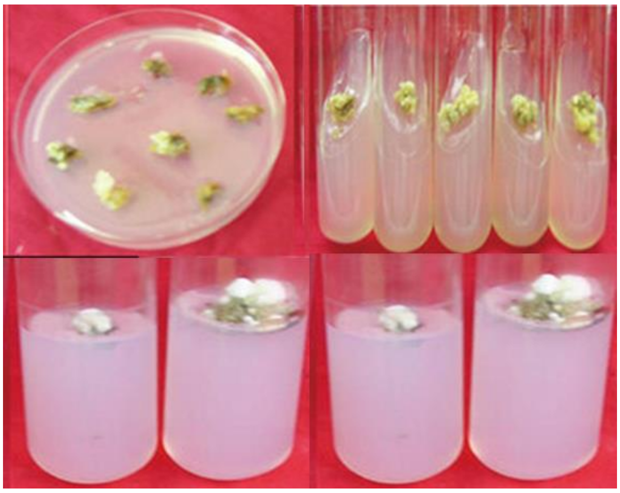

Fig. 6: Callus induction in callus inducing medium.

Micro Propagation

The callus produced in the tubes containing callus inducing medium was then transferred into different tubes for micro propagation. The emergence of plantlets was seen in the tubes (Fig. 7).

Fig. 7: Emergence of plants in tubes containing MS medium.

Shifting of Plants into the Pots

The plants were then shifted to the pots placed in an acclimatization room. It was observed that some of the plants were survived whereas rest of the plants were died (Fig. 8a, 8b).

Fig. 8 (a): Shifting of plants in acclimatization room.

Fig. 8 (b): Shifting of plants in acclimatization room.

Biomass Measurement

Fresh biomass was measured daily; the callus samples were weighed from the culture medium. Initial fresh weight was 15g and treatment with humic acid began at day 1 at a level of 2g and was progressively raised to 19g at day 5. The stock of the fresh biomass was also gradually increasing during the entire period of the research, pointing at its favorable growth tendencies. Determination of dry biomass was done by weighing the callus samples and placing them in an oven at a given temperature and the weight taken till a constant weight was obtained. The average of the dry weight increased from 3.8g on Day 1 to 5.1g on Day 5 while the control group received only 1g of the same on the same day. The amount of water in the callus was obtained by determination of the fresh and dry mass of the tissue. Within the study period it ranged between 11-17%. The effect of humic acid on callus formation, however, was further evaluated concerning the daily growth rate. By Day 5, a 25% increase of 4% in biomass was recorded with the humic acid treated samples as compared to the control as shown in the Table 1.

Table 1: Indicating the changes in fresh and dry biomass

Sr. No.	Measurement type	Units	Day 1	Day 2	Day 3	Day 4	Day 5
1.	Fresh biomass	g	15.2	16.0	17.1	18.3	19.3
2.	Dry biomass	g	3.8	4.1	4.3	4.7	5.1
3.	Water contents	g	11.4	11.9	12.8	13.6	14.2
4.	Growth dynamics	%	0	5.3	12.5	20.2	25.4

Evaluation of Humic Acid Inducing Cotton Plants for Resistance against Cotton Bollworm (*H. armigera*)

The leaves of humic acid inducing and non-inducing plants were selected from for bioassay analysis. Leaves from both plants were exposed to cotton bollworm. The 5–7, 3rd instar larvae were used in triplicates, and infestation data were recorded. On the third day of infection, 60% mortality was observed against cotton bollworm in humic acid inducing plants, whereas no mortality was observed in non-humic acid inducing plants (Fig. 9).

Fig. 9: Insect bioassay on plant leaves containing humic acid.

DISCUSSION

The main focus of this study was to shed light on the response of cotton (*Gossypium*) to humic acid treatments, against pesticide of cotton bollworm (*Helicoverpa armigera*), results focused on the germination, callus formation, micro propagation and resistance to cotton bollworm (*H. armigera*). Results indicated the successful germination of cotton seeds after five-minute treatment with 0.1% sodium hypochlorite solution and incubation after 24 hours. The previous study also observed the germination of cotton seed after surface sterilization at the day 2 of the experiment that showed the consistency with the results of our research in which the germination took place after 24h also (Maeda et al., 2021). The basic substrate, such as 1/2 MS medium which has been proven to successfully germinate and grow cotton and similar results has been administrated in the previous study in that the use of MS medium highly improved germination rates and plantlet development in cotton as suggested by them (Bakhsh et al., 2016). The germination has been considered as the base of the robust plant systems for studies. The sterilization and media preparation protocol were proved by observing that the embryos could germinate within 24h in this research. This finding was supported by previous studies where fast germination was obtained under favorable conditions (Hu et al., 2015).

In plant tissue culture, induction of callus from leaf explants using MS medium supplemented with auxins (2,4-D) and cytokinin in a well-established technique used widely. The previous study reported a slight increase in callus production with some of these growth regulators and our results supported the findings that those related substances were effective on callus formation as mentioned by Wen et al. (2020) developments. Callus induction as observed in our study, is one of the widely employed techniques in plant tissue culture research and here in auxins and cytokinin are extensively used to promote cellular proliferation and dedifferentiation (Wen et al., 2020; Zafar et al., 2022).

Our study also suggested a novel role of humic acid in callus formation. The previous studies also found that humic acid could enhance callus growth and regeneration in several plant species. Supplementation of humic acid to our media form in rising induction and development of callus indicating it benefiting role by providing essential nutrients or some growth factors required for cellular activity mechanisms (Shahrajabian & Sun, 2024). Another study in wheat crop plant has similar procedure that confirmed the induction of callus through humic acid treatment (Arslan et al., 2021). These findings showed consistency with the results of this study that the humic acid result in the induction of callus in cotton crops. The present investigation is also useful in understanding the habitat of humic acid uses besides general plant growth and clearly indicates that applications of HA can help overcome tissue culture problems. The key to further studies or the agricultural production of uniform plantlets is micro- propagation made possible by efficient induction of callus. This research indicated that the plantlets developed from callus cultures is in accordance with previous reports where micro-propagation had been done using protocols similar to which has been employed in this work. This successful transfer of plantlets to acclimatization conditions has been another indication that humic acid applications included in the processes related with plant propagation system (Afolabi-Balogun et al., 2015).

The results of this study revealed increased resistance in humic acid-treated cotton plants to both the biological characteristics and development rate on the larvae of a major lepidopteran pest, cotton bollworm (*Helicoverpa armigera*). The mortality rates were even higher in humic acid-treated plants (60% compared to 0 controls). Although humic acid treatment has been desirable trait with a potential significant increase in resistance influence. The idea that humic acid promoted plant immunity is consistent with findings from previous researches. The application of humic acid can increase pest resistance in several crops. These effects of age on the increase in resistance that has been observed due to combination of several properties. Humic acid could induce stress in the plant alters its secondary metabolome, so that it assumes a defensive position against pests. Humic acid increases the nutrient availability which helps the plants grow healthier and able to resist better that showed the uniformity with this study results (Matuszak-Slamani et al., 2017; Nasab et al., 2019).

Results from this study differ significantly compared to some studies where no effects of humic acid on pest resistance have been detected. Such discrepancy could lie in various experimental conditions, plant varieties and pest species. Further studies are needed to explore the underlying mechanisms by which humic acid acts a plant resistance inducer, and its interaction with other growth regulators or environmental factors. The final results of this study are in accordance with the previous findings about humic acid and its ability to affect growth as well pest resistance. As having been reported in earlier years, MS medium increased plant growth promotion ability as well as tissue culture efficiency rather than the other media at least for some study which was then confirmed by our observation. Furthermore, our results lend support to the body of evidence which suggested a wide array of positive benefits from use in agriculture for humic acid. The humic acid treatment induced the bollworm host susceptibility (mildly to moderately susceptible) to become mildly resistant, offering a new potential application. While studies have recently provided substantial evidence for the effects of humic acid on plant biomass and growth, as yet this research embodied an application not only looking at its potential to enhance pest resistance, but also in a commercial context such field trials rarely assess implications beyond direct bioavailability studies or classical agricultural performance tests. Their discovery implies that humic acid could become an optimal agent for integrated pest management (IPM) strategies (Pereira et al., 2019).

Our results have monumental agricultural implications, especially in the case of cotton farming. These results indicated that adding humic acid in a crop system may enhance pest resistance and reduce reliance on chemical pesticides. In the future, it would be interesting to research how these responses are modulated by humic acid in maize plants. Such findings suggested that further research should investigate the effects of humic acid on secondary metabolites, gene expression related to induced defense responses in plants, and also its interactions with other growth regulators. Further studies need to be conducted, especially for the long-term effect of humic acid on plant economy, output and environmental sustainability. Conclusively, our findings solidified and expanded upon previous studies by indicating the ameliorative effects of humic acid on cotton germination, callus induction with respect to resistance against cotton bollworm (*Helicoverpa armigera*). These results added to the mounting body of evidence in favor of including humic acid as a crop enhancement which would make them more robust crops under abiotic stress and also served some role in integrated pest management (Fan et al., 2021).

Conclusion

Cotton (*Gossypium*) is a crucial crop with significant agricultural and economic importance. Enhancing its resistance to pests like *Helicoverpa armigera* through effective treatment strategies is vital for sustainable cotton farming. The study highlighted a strong perspective for the role of humic acid as growth promoter and pest defense component in cotton (*Gossypium*) plants. The results indicated that the application of humic acid is not only beneficial to seed germination and callus formation, but also conducive to improve the comprehensive resistance of cotton bollworm (*Helicoverpa armigera*) in cotton. The structural difference between control and treatment groups were indicated that the incorporation of humic acid into the growth medium resulted in significant increases both the rate and efficiency of callus induction, as well a significantly increased pest resistance about 60% mortality with bollworm larvae for treated plants versus no mortality recorded at all on any untreated plant. These findings indicated that humic contributed to the promotion of cotton productivity by reinforcement in plant defensive mechanisms against pests, which led to a decrease in chemical pesticide use. The beneficial effects on callus formation and the growth in vitro of plants also sustain the practical utility hamates for tissue culture methods to mass propagation. Further investigations are necessary to explore the molecular mechanisms that underlie humic acid-mediated modulation of plant defense and how it interacts with other growth-regulatory hormones. Besides, long-term study on the cotton yield and additional advantages of using humic acid in integrated pest management system might provide more points. Thus, this study added to a growing body of evidence supporting the positive utility and impact of humic acid in agriculture broadly for enhanced crop resistance (plant immunity) and sustainability.

Funding: This study did not get any financial support from any organization.

Conflict of Interest: The authors declare no conflict of interest.

Consent for Publication: All authors declare their consent for publication in this journal.

Author's Contribution: AG, FQ, and AR wrote the initial draft of the manuscript. GR, MMZ, AR and SS provided the space and helped to experiment. AH, MSC, MAK, AA and VS Conceptualization, writing—review and editing; HB, and NA data curation and helped in statistical analysis. RA, WJ, PA, FM, MH, AS and SMR helped in writing-review and editing, and FQ and MMZ reviewed and supervised the experiment. All authors approved final version of the manuscript.

Data Availability: The data is available from the corresponding author(s) based upon a reasonable request.

Generative AI statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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