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APPLICATION OF *ASPERGILLUS ORYZAE* ASU44 (OL314732) AND THEIR KOJIC ACID AS PESTICIDES AGAINST COTTON APHID, *APHIS GOSSYPII*

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Because of their negative effects on the environment, humans, and other organisms, chemical pesticides have been prohibited in many countries. Alternative microbial management is easy-to-implement, effective, and safe for humans and the environment. Fungus-based bio-pesticide products like kojic acid could commercialize as effective alternative chemical pesticides. Aspergillus oryzae ASU44 (OL314732) was isolated from hollyhock rhizosphere and identified using 18S rRNA gene sequencing. The strain showed high ability of kojic acid production especially after optimizing the production using 41-run Box-Behnken statistical design. Maximum kojic acid production was 46.53 g/l (predicted values 46.62 g/l) obtained in run number (23) using Glucose (150, g/l), Yeast extract (5 g/l), KH_2PO_4 (3 g/l), $MgSO_4.7H_2O$ (0.5 g/l) and pH (3). The design was effective to applied with coefficient (R^2) 0.988, 0.986 (adjusted R^2 0.975, 0.972) for kojic acid (g/l) and dry fungal mass formation (g/l). The pathogenicity of the fungus and their kojic acid in addition to standard synthetic kojic acid was investigated to the cotton aphid, Aphis gossypii Glover (Homoptera: Aphididae). All treatments had a considerable impact on aphid mortality. The mortality rate was relative to the duration of exposure and the quantities of bio-pesticides agents. However, data revealed that as concentrations increased, the lethal time values decreased. Aspergillus oryzae ASU44 and its extract containing kojic acid were found effective against A. gossypii. As a result, this research suggests that these agents could be effective in aphid cotton management.

Keywords: *Insecticides. Aphid management. Aphis gossypii. Aspergillus oryzae. Kojic acid.*

INTRODUCTION

Aphids (Hemiptera) are common agricultural pests that inhabiting temperate and tropical climates. Aphids cause direct and indirect damage to world food and fiber crops by ingesting phloem sap and spreading viruses and other infections. Feeding and excreting honeydew encourages the development of fungi that clog leaf stomata, reducing photosynthesis[1].Consequently, plant growth is inhibited which has a detrimental influence on yield[2]. The cotton-melon aphid, *Aphis gossypii* is a polyphagous insect found in tropics, subtropics, and temperate zones. It is harmful to cotton plants and many other plant species such as okra and eggplants. This aphid assaults can diminish leaf area, biomass, branching and plant height, all of which can affect the crop and result in financial losses[3].

Aphids have been combated using a variety of methods. The usually utilized tactics in insects control are the physical and chemical ways. To date, a range of chemical pesticides have been applied in the treatments against insects. Consequently of the increased use of pesticides to achieve sufficient control; environmental degradation, disruption of the

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natural ecological balance and human health issues has increased[4&5]. As a result of its ubiquitous use of insecticides, certain aphid species have developed resistance. Thus, strong population management is essential[6]. Alternatively, study of biological aphid control is still ongoing. Many microbial pesticides based on pathogenic organisms including virus, bacteria, fungi and nematode have played a crucial function in crop protection and are now employed to control a wide spectrum of insects[7].

Insects can be infected by fungi, which can cause symptoms like as reduced feeding, delayed development, decreased mating success and even death[8]. Kojic acid ($C_6H_6O_4$) is a natural organic acid with the chemical formula 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one and it is a secondary metabolite biologically produced by various types of fungi during the aerobic fermentation of various foods[9]. It's popular in the cosmetics and health-care businesses especially in skin whitening creams, whitening soaps, skin protection lotions, and teeth care products[10]. It has the ability to serve as an ultra violet defender, suppressing hyperpigmentation in human skins by inhibiting the development of melanin by inhibiting the formation of tyrosinase, the enzyme responsible for skin pigmentation[11].

Kojic acid mainly produced by some fungal genera especially *Aspergillus*, *Mucor* and *Penicillium*, however the most common species are *A. oryzae*, *A. flavus*, *A. tamari*, and *A. parasiticus*[12-15]. Through the action of cell enzymes, kojic acid is biologically synthesized directly from glucose (which serves as a precursor). Glucose-6-phosphate dehydrogenase, gluconate dehydrogenase, and hexokinase are among the cell-bound enzymes involved in the direct synthesis of kojic acid from glucose[16&17]. When interactions between different components are present, the traditional way of medium optimization, one factor at a time, is time consuming, expensive, and frequently leads to misinterpretation of results. Statistical experimental designs reduce the amount of error in determining the influence of parameters and allow for simultaneous, systematic, and efficient manipulation of all variables[18-20].

Antimicrobial characteristics of kojic acid make it useful in a variety of sectors. Kojic acid, according to Beard and Walton[21], can be used as an insecticide and pesticide. In the manufacturing of pesticides, kojic acid and its derivatives have the potential to be utilized as a chelating agent[22]. However, few studies on kojic acid's pesticidal activity have been published a long way. The purpose of this study was to enhanced kojic acid production by *Aspergillus oryzae* ASU44 (OL314732) and see how the fungus and its secondary metabolite, kojic acid, affected *A. gosypii* as important cotton pest.

MATERIALS AND METHODS

Fungal isolation and identification

Aspergillus oryzae ASU44 was isolated from hollyhock rhizosphere on Czapek's dextrose agar (CzDA) medium at 28 ± 1 °C, and primary tested for kojic acid production using ferric chloride reagent which produce red color around the fungus. Based on its macroscopic and microscopic features, the fungus was first identified as *A. oryzae*. Then, using the universal primers ITS1 and ITS4, they were genetically identified[23&24]. A small portion of 4-day-old fungal growth of *Aspergillus oryzae* ASU44 grown on CzDA plates at 30 ± 1 °C were collected, transferred to tube contains CTAB buffer and incubated at 65 ºC for 30 min. Isopropanol was added for the DNA precipitation, the pellets were washed with ammonium acetate buffer. After centrifugation at 13,000xg for 10 min, the supernatants were discarded and the pellets suspended in distilled water for PCR and sequencing in SolGent, Daejeon, Korea according to White (1990)[23]method. The sequences were evaluated and compared to sequences from closely related species before being placed in the GenBank database with a unique entry number.

Testing for aflatoxins production by *A. oryzae* ASU44

Aspergillus oryzae ASU44 growing in flask had sterilized potato dextrose broth medium (200 g/l potato and 20 g/l dextrose in 1 L water) and incubated at 30±1°C for 10 days was tested for its ability to produce aflatoxins. Culture flask were homogenized with chloroform (1:1, v:v) for 5 min, filtrate to remove the mycelia, then the organic layer was

separated using separation funnel and evaporated using water bath to 1 ml. Thin layer chromatography (TLC) was used for aflatoxins detection on silica gel plate (60 GF254) injected with 100 µl extract and 100 µl authentic aflatoxins, put in chamber contains chloroform: acetone (90:10, v:v) as solvent system. The developed plate let to dry and examined in two ultraviolet radiation wave lengths 254 and 356 nm.[20&25].

Enhancing kojic acid production using Box-Behnken statistical design

Aspergillus oryzae ASU44 was aerobically grown on Czapek's dextrose agar plates (dextrose 20; $NaNO_3$, 3.0; KH_2PO_4, 1.0; KCl, 0.5 and $MgSO_4 \cdot 7H_2O$, 0.5) at 30 ± 1 °C for 3 days, then fungus spores were suspended in sterilized 0.1%, Triton X100 (v/v) and diluted to final concentration 2×10^5 spores/ml. Modified Czapek's dextrose broth medium was utilized as fermentation medium (g/l): dextrose 100; yeast extract, 5.0; KH_2PO_4, 1.0 and $MgSO_4 \cdot 7H_2O$, 0.5, dissolved in 1L distilled water with initial pH 3 and autoclaving at 1.5 atmospheric pressure, for 20 min at 121 °C. After that the medium was inoculated with 2% inoculum (2×10^5 spores/ml) and incubated at 30 ± 1 °C, 150 rpm rotatory shaker for one week[15&20].

Box–Behnken experimental design was used for enhancing kojic acid production by *Aspergillus oryzae* ASU44 *via* studying the interactions between different parameters, the main effects, and quadratic effects of the evaluated variables. Five parameters, 3-levels randomized statistical design with forty-one runs were conducted exploring. Glucose (g/l) (A), yeast extract (g/l) (B), KH_2PO_4 (g/l) (C), $MgSO_4 \cdot 7H_2O$ (g/l) (D) and pH (E) were stated by three levels low, medium, and high concentrations (-1, o, +1). The non-linear quadratic statistical model was generated by the quadratic equation (1) as cleared by Liu (2013)[26] and Yan (2014)[27].

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_{ij} \quad (1)$$

Y stands for the projected values of kojic acid, and dry mass of *Aspergillus oryzae* ASU44, β_0 is the intercept, β_i is the linear impact, β_{ii} is the squared impact, β_{ij} is the interaction impact, x_{ij} is the independent levels of variables. Response surface plots, curves of actual and expected values, and statistical analysis of all data were used to clarify the variables' linkages. For the validity of the created models, Derringer's required equations were set. Data were analyzed using Design Expert 7.0.0 statistical software from the United States, which included quadratic regression and variable interaction analysis using one-way ANOVA with a P value of 0.05.

Kojic acid quantification

After incubation; the mycelia were filtrated on weighted filter paper and transferred to hot air oven at 60 ºC until constant weight for fungal dry mass estimation. Supernatants were used for determination of both kojic acid and residual sugars. Kojic acid was determined following Bentley (1957)[28], and Mahmoud and Zohri (2021)[20]: where 1 ml of diluted sample was mixed with 1 ml of ferric chloride ($FeCl_3$) reagent prepared in HCl (0.1N). The reaction between the functional group of hydroxyl in the samples and $FeCl_3$ produced red color, measured at wavelength 540 nm using spectrophotometer. Kojic acid values then calculated from KA 0.1 – 2.0 mg/ml standard curve. Residual sugar determined using dinitrosalicylic acid (DNS) method as described by Miller[29]. Absorbance measure using spectrophotometer at wavelength 540 nm. Residual sugar values then calculated from glucose 1 - 10 g/l standard curve.

Effect of *Aspergillus oryzae* ASU44, biological kojic acid and synthetic kojic acid on cotton aphid

Aphid rearing

Cotton aphid, *A. gossypii* infected leaves were gathered from okra plants planted at Assiut University's experimental farm. Before the experiment, the aphid populations were reared on okra plants in a laboratory incubator with a temperature of 22±2 ºC, a relative humidity of 65±5%, and a photoperiod of 16:8 (light: darkness) [30]. In order to produce a homogeneous population, the aphid colony was kept alive for numerous generations. Aphids were collected from subsequent generations for bioassay investigations[31].

Solutions preparation

Five concentrations were prepared from stock solutions of the used agents for bioassay. Serial inoculum concentrations of the fungal strain, *Aspergillus oryzae* ASU44 (0, 2.5×10^3, 5×10^3, 7.5×10^3 and $1x10^4$ spores/ml) were prepared. The used concentrations from the fungal extracted kojic acid were (0. 25, 50, 75 and 100 ppm). As well as, the same five concentrations of the standard synthetic kojic acid were used.

Bioassay and pathogenicity

Under laboratory conditions, the pathogenicity of all the prepared solutions as treatments on aphids was assessed using a Petri dish experiments. Ten healthy aphids were placed into plastic Petri dishes (10 cm diameter * 1.5 cm height) with okra plant leaves. Fresh leaves from the tops of okra plants were used to aphid feeding. Before feeding, the leaves were cleaned with tap water and air dried. A layer of wet filter sheets was used to line each Petri dish. To keep the leaf fresh and retain humidity, it was wrapped in wet cotton and bathed in water on a daily basis [32&33]. The virulence of the tested insect strain was then estimated, aphids were treated using pre-made solutions and placed in okra leaves on Petri plates. As a control, untreated aphids were given merely distilled water[30]. Each infection was carried out five times in total. All of the dishes were reared in laboratory chambers. After that, observations were checked every 24 hours and mortality rates of aphids were calculated[34]. When there is natural mortality among the controls, Abbott's formula **(Abbott, 1925)** was used to compute aphid mortalities.

Abbott's mortality (%) = {(Living number of aphids in control) – (Living number of aphids in treatment)/ (Living number of aphids in control)} ×100

Statistical analysis

Using SPSS version 16.0 software, the acquired data were analyzed using Duncan's multiple range at 0.05% (SPSS INC, Chicago, IL). To achieve the LC_{50} and LC_{90} values with 95 percent confidence intervals, data were submitted to probity analysis employing filler's approach[36] through a computer program. As well as, lethal time values (LT_{50} and LT_{90}) were estimated. By comparing the tested solutions, the efficacy of various solutions was determined.

RESULTS AND DISCUSSION

Results

Fungal isolation and identification

On Czapek's dextrose agar (CzDA) medium at 28 ± 1 °C, *Aspergillus oryzae* ASU44 was isolated from hollyhock rhizospher and molecularly identified using the universal primers ITS1 and ITS4. ITS sequencing of *Aspergillus oryzae* ASU44 showed high similarity (100%) with GenBank accession numbers *Aspergillus oryzae* (Accession no. MK714921.1), *A. oryzae*, (Accession no. MK163533.1), *A. oryzae* (MG437005.1), and *A. oryzae* (MH329786.1). It is worth to mention that this fungal strain could not to produce aflatoxins in PD broth under optimum growth conditions and identified as *Aspergillus oryzae* ASU44 (Accession no. OL314732).

Enhancing kojic acid production using Box-Behnken statistical design

The interaction effects, main effects, and quadratic effects of the studied variables on the synthesis of kojic acid by *Aspergillus oryzae* ASU44 (OL314732) in glucose fermentation medium were optimized and evaluated using a Box–Behnken statistical experimental design. The strain was deemed to be safe and did not produce aflatoxins. Five-parameter (Glucose (100, 150, 200 g/l) (A), Yeast extract (1, 5, 10 g/l) (B), KH_2PO_4 (0, 1, 3 g/l) (C), $MgSO_4.7H_2O$ (0, 0.5, 2 g/l) (D) and pH (3, 5, 7) (E)), three-level (-1, 0, +1) statistical design with 41-runs was established as mentioned in table (1). The predicted values of the completed runs were determined using the equation (1) for second-order polynomial; the predicted values of kojic acid (g/l) and dry fungal mass (g/l) were computed using the second-order polynomial equation (1); kojic acid (g/l) and dry fungal mass (g/l) predicted values calculated by the equations (2 &3) as following:

Table 1: Box-Behnken design with five variables; Glucose (g/l) (A), yeast extract (g/l) (B), KH_2PO_4 (g/l) (C), $MgSO_4 \cdot 7H_2O$ (g/l) (D) and pH (E) with actual and/or predicted responses of kojic acid (g/l) (KA) and dry mass (g/l) (DM) by *Aspergillus oryzae* ASU44 (OL314732).

Trials	Glucose (g/l)	Yeast extract (g/l)	KH_2PO_4 (g/l)	$MgSO_4 \cdot 7H_2O$ (g/l)	pH (E)	Actual values of KA (g/l)	Predicted values of KA (g/l)	Actual values of DM (g/l)	Predicted values of DM (g/l)
1	1	-1	0	0	0	29.84	29.41	4.18	3.92
2	0	1	0	0	1	35.29	34.67	14.97	15.35
3	1	0	0	0	1	29.69	30.22	7.04	7.74
4	0	1	1	0	0	34.80	35.05	15.62	15.73
5	0	-1	0	1	0	29.93	29.23	4.66	4.64
6	0	0	-1	1	0	26.67	27.09	9.82	10.02
7	0	-1	0	0	1	27.16	27.77	4.34	3.63
8	0	-1	1	0	0	29.00	29.53	4.64	5.32
9	1	1	0	0	0	34.93	36.04	17.72	17.32
10	-1	0	0	0	-1	31.49	30.74	8.47	7.99
11	0	0	-1	-1	0	30.28	29.28	8.80	9.31
12	0	1	0	-1	0	38.39	39.00	16.29	16.39
13	0	0	1	0	1	19.61	19.69	9.11	8.36
14	0	0	-1	0	-1	14.92	15.43	8.14	8.69
15	-1	1	0	0	0	33.17	34.01	15.02	15.44
16	0	-1	0	0	-1	30.31	30.60	4.74	4.08
17	-1	0	0	-1	0	36.63	36.91	9.67	9.31
18	1	0	0	-1	0	34.75	35.06	10.51	10.26
19	0	1	0	1	0	37.51	36.77	18.62	18.22
20	1	0	1	0	0	34.09	33.45	8.06	8.75
21	0	0	0	-1	-1	35.09	35.87	9.25	9.35
22	1	0	-1	0	0	26.13	26.27	9.48	9.79
23	0	0	1	0	-1	46.53	46.62	9.18	9.22
24	0	0	0	1	-1	36.51	37.54	9.79	10.52
25	-1	0	-1	0	0	24.20	24.39	8.32	7.69
26	0	1	-1	0	0	29.87	29.35	17.56	16.92
27	0	0	0	-1	1	38.96	37.89	9.82	9.35
28	-1	0	0	1	0	29.95	29.90	10.23	10.04
29	0	-1	0	-1	0	32.40	33.05	5.30	5.78
30	0	0	0	1	1	31.00	30.19	8.71	8.87
31	0	0	1	1	0	32.33	33.19	10.56	10.14
32	-1	0	0	0	1	31.36	32.15	7.71	8.65
33	0	0	1	-1	0	37.60	37.03	10.26	10.15
34	-1	-1	0	0	0	27.87	27.16	4.11	4.66
35	1	0	0	0	-1	37.96	36.95	10.76	10.04
36	-1	0	1	0	0	31.64	31.05	9.95	9.70
37	0	-1	-1	0	0	21.63	21.39	3.22	3.15
38	0	0	0	0	0	29.07	29.07	10.36	10.36
39	0	0	-1	0	1	36.53	37.03	8.16	7.92
40	1	0	0	1	0	36.04	36.03	10.30	10.23
41	0	1	0	0	-1	38.12	37.18	16.11	16.54

Kojic acid (g/l) = 29.07+(1.07) A +(3.37) B + (3.46) C + (-1.51) D +(-1.33) E + (-0.0533) AB + (0.13) AC + (1.99) AD + (-2.03) AE + (-0.61) BC + (0.3967) BD + (0.08) BE + (-0.4133) CD + (-12.13) CE + (-2.34) DE + (1.27) A^2 + (1.31) B^2 + (-1.55) C^2 + (4.13) D^2 + (2.17) E^2 (2)

Dry mass (g/l) = 10.36+(0.286) A +(6.05) B + (0.243) C + (0.174) D +(-0.411) E + (0.658) AB + (-0.763) AC + (-0.193) AD + (-0.74) AE + (-0.84) BC + (0.743) BD + (-0.185) BE + (-0.18) CD + (-0.023) CE + (-0.413) DE + (-0.661) A^2 + (0.637) B^2 + (-0.715) C^2 + (0.261) D^2 + (-1.1) E^2 (3)

Maximum experimental values of kojic acid production was 46.53 g/l; whereas the corresponding predicted values was 46.62 g/l (dry mass 9.18 g/l (predicted 9.22 g/l)) obtained in run number (23) using Glucose (150, g/l) (A), Yeast extract (5 g/l) (B), KH_2PO_4 (3 g/l) (C), $MgSO_4.7H_2O$ (0.5 g/l) (D) and pH (3) (E), however the lowest kojic acid production cleared was 14.92 g/l; whereas the corresponding predicted values was 15.43 g/l (dry mass 8.14 g/l (predicted 8.69 g/l)) obtained in run number (14) using Glucose (150, g/l) (A), Yeast extract (5 g/l) (B), KH_2PO_4 (0 g/l) (C), $MgSO_4.7H_2O$ (0.5 g/l) (D) and pH (3) (E) as cleared in in table (1).

The predicted values of kojic acid production and fungal dry mass were found to be very near to the response model experimental data, indicating that the established model is accurate as cleared in Table 1 and figures (1a&b). The statistical model suitability, accuracy, and significance of the quadric polynomial equations (2 & 3) were further examined using one-way analysis of variance to corroborate the statistical model suitability, accuracy, and significance (ANOVA) as established in table 2. The *F* and *P*- values of the tested model of kojic acid production (g/l) and dry fungal mass (g/l) (*F;* 79.52, 71.09 and *P*; <0.0001 for each, respectively) indicating that the parameter yielded significant results (Probability ≤0.05). The statistical parameter coefficient (R^2) was also determined to evaluate the model goodness, accuracy, and fitting; R^2 values of kojic acid production (g/l) and dry mass (g/l) were 0.988, 0.986 and adjusted R^2 value 0.975, 0.972 which indicated that the whole variations were explained highly by the statistical model.

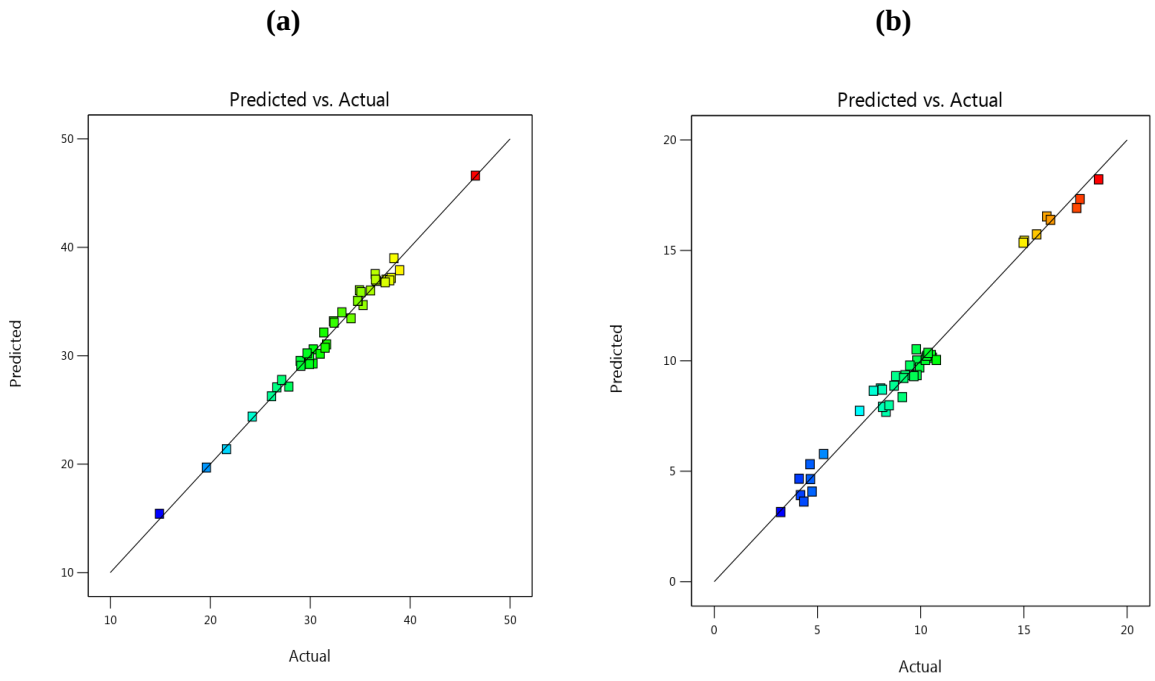

Fig. 1: Comparison between the actual and predicted values of kojic acid (g/l) production (a) and dry mass (g/l) (b) by *Aspergillus oryzae* ASU44 (OL314732).

The significance and effects of each individual variable and/ or the interactions were set in table (2) as one-way ANOVA results. Individual variables of Glucose (A), Yeast extract (B), KH_2PO_4 (C), $MgSO_4.7H_2O$ (D) and pH (E) have significant effects on kojic acid production. The interaction between different variables AD (Glucose (g/l) * $MgSO_4.7H_2O$ (g/l)), AE (Glucose (g/l) * pH), CE (KH_2PO_4 (g/l) * pH), and DE ($MgSO_4.7H_2O$ (g/l) * pH) were found to be ($P < 0.05$) significant for kojic acid production (g/l), while the interaction between AC (Glucose (g/l) * KH_2PO_4 (g/l)), AE (Glucose (g/l) * pH), BC (Yeast extract (g/l) * KH_2PO_4 (g/l)), and BD (Yeast extract (g/l) * $MgSO_4.7H_2O$ (g/l)) were significant for dry mass (g/l).

Table 2: ANOVA results for Box-Behnken quadratic model of kojic acid (g/l) and dry mass (g/l) (DM) by *Aspergillus oryzae* ASU44 (OL314732).

	Sum of Squares		Mean Square		*F*-value		*P-value*	
Source	KA (g/l)	DM (g/l)	KA (g/l)	DM (g/l)	KA (g/l)	DM (g/l)	KA (g/l)	DM (g/l)
Model	1312.66	628.40	65.63	31.42	79.52	71.09	< 0.0001	< 0.0001
A-Glucose (g/l)	18.35	1.31	18.35	1.31	22.23	2.95	0.0001	0.1011
B-Yeast extract (g/l)	181.89	584.67	181.89	584.67	220.37	1322.89	< 0.0001	< 0.0001
C-KH_2PO_4 (g/l)	191.73	0.9409	191.73	0.9409	232.29	2.13	< 0.0001	0.1601
D-$MgSO_4.7H_2O$ (g/l)	36.48	0.4865	36.48	0.4865	44.20	1.10	< 0.0001	0.3066
E-pH	28.41	2.71	28.41	2.71	34.42	6.12	< 0.0001	0.0224
AB	0.0114	1.73	0.0114	1.73	0.0138	3.91	0.9077	0.0619
AC	0.0676	2.33	0.0676	2.33	0.0819	5.26	0.7777	0.0328
AD	15.89	0.1482	15.89	0.1482	19.26	0.3354	0.0003	0.5690
AE	16.54	2.19	16.54	2.19	20.04	4.96	0.0002	0.0377
BC	1.49	2.82	1.49	2.82	1.80	6.39	0.1944	0.0200
BD	0.6294	2.21	0.6294	2.21	0.7625	4.99	0.3929	0.0371
BE	0.0256	0.1369	0.0256	0.1369	0.0310	0.3098	0.8620	0.5840
CD	0.6834	0.1296	0.6834	0.1296	0.8280	0.2932	0.3737	0.5941
CE	588.87	0.0020	588.87	0.0020	713.45	0.0046	< 0.0001	0.9467
DE	21.96	0.6806	21.96	0.6806	26.61	1.54	< 0.0001	0.2290
A^2	5.03	1.35	5.03	1.35	6.10	3.06	0.0227	0.0956
B^2	5.35	1.26	5.35	1.26	6.48	2.84	0.0193	0.1075
C^2	7.45	1.58	7.45	1.58	9.02	3.58	0.0070	0.0730
D^2	52.86	0.2107	52.86	0.2107	64.05	0.4767	< 0.0001	0.4979
E^2	14.65	3.72	14.65	3.72	17.75	8.43	0.0004	0.0088

Response surface plots and the interaction plots draw for the 3D visualization of the cleared interaction between pair-wise of the two factors when the other factor constant as showed in figures (2-4) explaining the effect of (Glucose (A), Yeast extract (B), KH_2PO_4 (C), $MgSO_4.7H_2O$ (D) and pH (E)) on kojic acid production (g/l) and dry mass (g/l) and reflect the interaction between AB (Glucose (g/l) * Yeast extract (g/l)), AD (Glucose (g/l) * $MgSO_4.7H_2O$ (g/l)), CD (KH_2PO_4 (g/l) * $MgSO_4.7H_2O$ (g/l)), AE (Glucose (g/l) * pH), CE (KH_2PO_4 (g/l) * pH), and DE ($MgSO_4.7H_2O$ (g/l) * pH) on kojic acid (g/l) production; AC (Glucose (g/l) * KH_2PO_4 (g/l)), AE (Glucose (g/l) * pH), CE (KH_2PO_4 (g/l) * pH), BD (Yeast extract (g/l) * $MgSO_4.7H_2O$ (g/l)) and DE ($MgSO_4.7H_2O$ (g/l) * pH) on dry mass (g/l) production by *Aspergillus oryzae* ASU44 (OL314732).

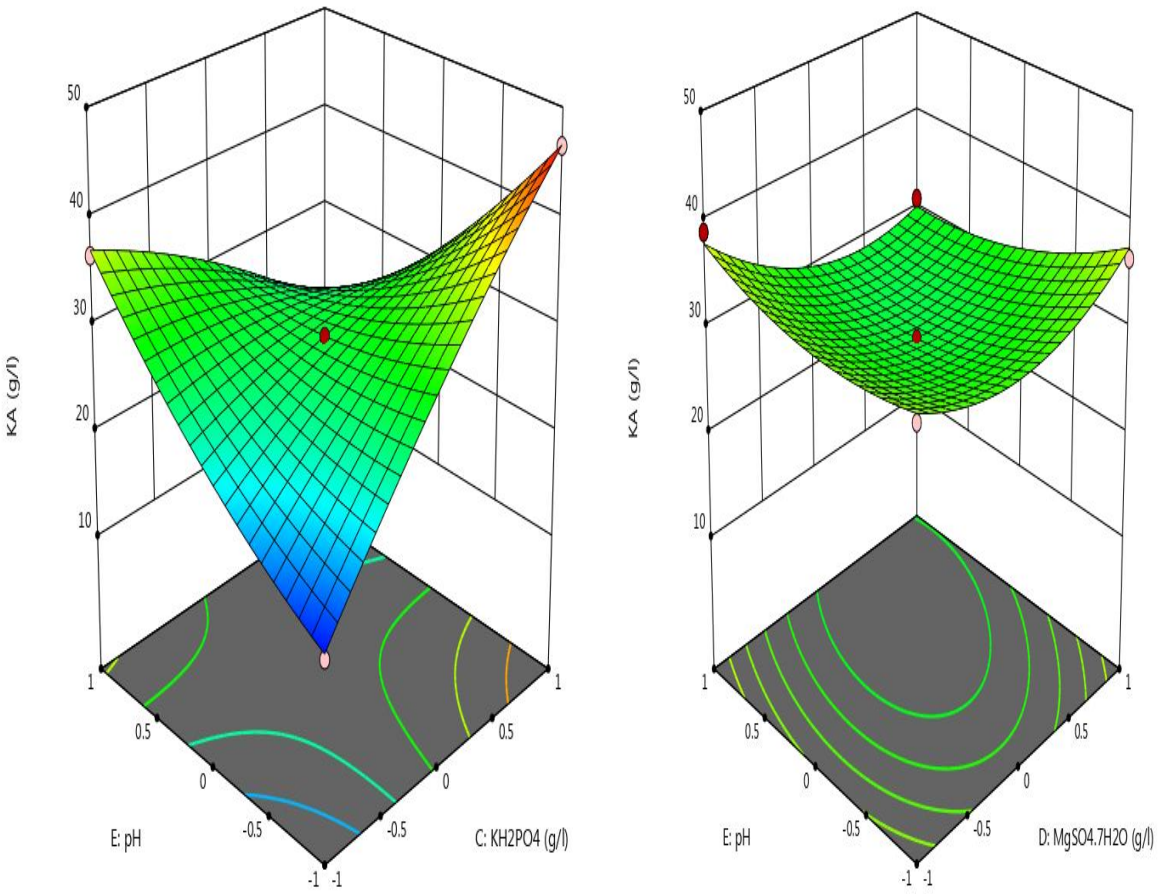

Fig. 2: 3D surface plots of Box–Behnken statistical experimental design explaining the interactions effects of AB (Glucose (g/l) * Yeast extract (g/l)), AD (Glucose (g/l) * $MgSO_4.7H_2O$ (g/l)), CD (KH_2PO_4 (g/l) * $MgSO_4.7H_2O$ (g/l)), AE (Glucose (g/l) * pH), CE (KH_2PO_4 (g/l) * pH), and DE ($MgSO_4.7H_2O$ (g/l) * pH) on kojic acid (g/l) production by *Aspergillus oryzae* ASU44 (OL314732).

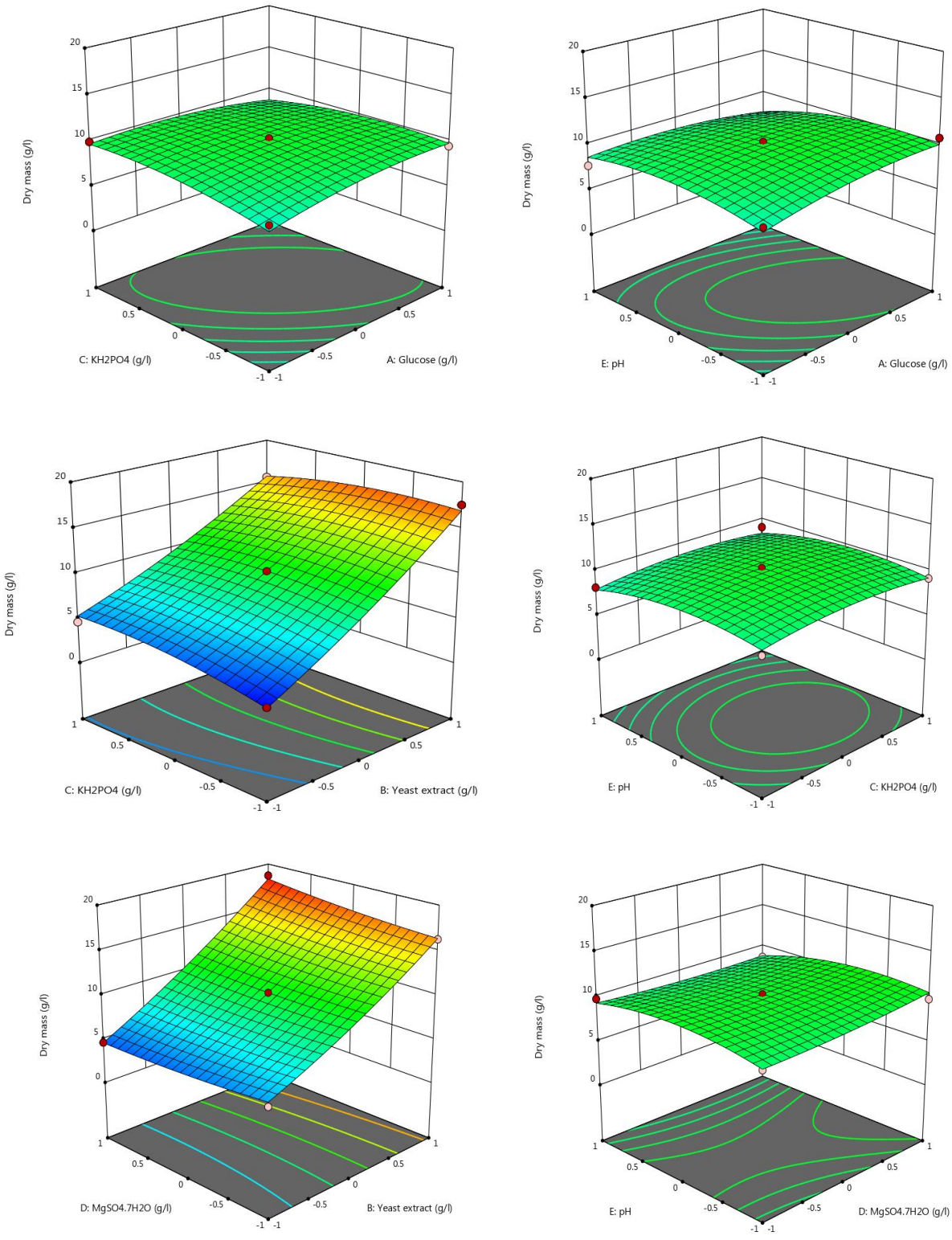

Fig. 3: 3D surface plots of Box–Behnken statistical experimental design explaining the interactions effects of AC (Glucose (g/l) * KH_2PO_4 (g/l)), AE (Glucose (g/l) * pH), CE (KH_2PO_4 (g/l) * pH), BD (Yeast extract (g/l) * $MgSO_4.7H_2O$ (g/l)) and DE ($MgSO_4.7H_2O$ (g/l) * pH) on dry mass (g/l) production by *Aspergillus oryzae* ASU44 (OL314732).

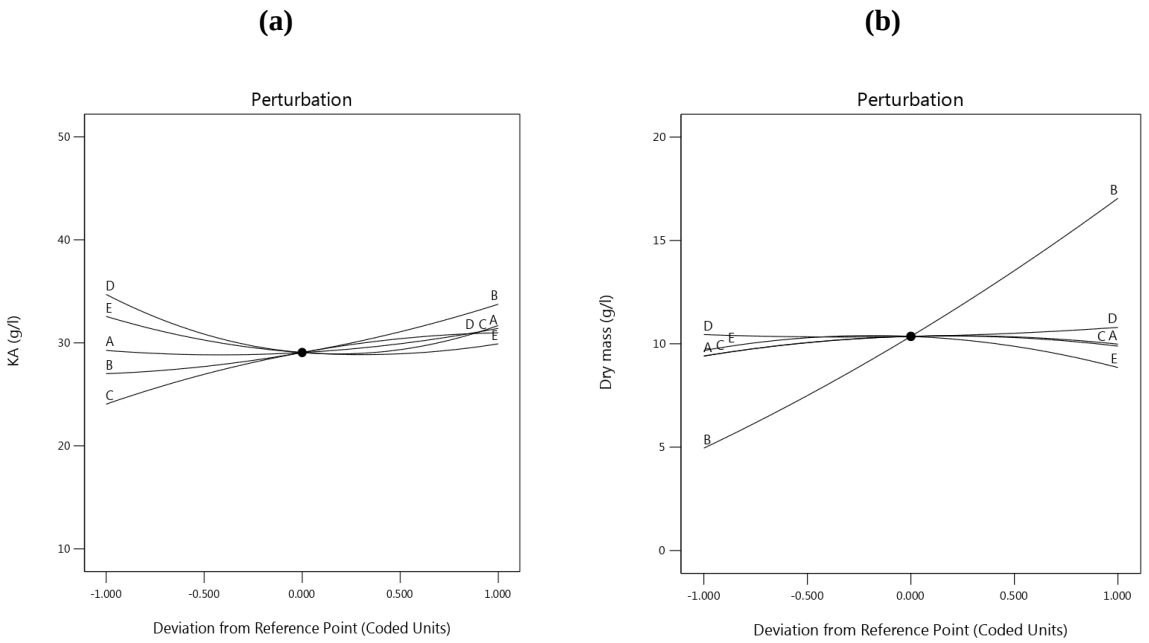

Fig. 4: The interaction plot of Box–Behnken statistical experimental design explaining the interactions effects of Glucose (g/l) (A), yeast extract (g/l) (B), KH_2PO_4 (g/l) (C), $MgSO_4 \cdot 7H_2O$ (g/l) (D) and pH (E) on kojic acid (g/l) production (a) and dry mass (g/l) (b) by *Aspergillus oryzae* ASU44 (OL314732).

The preceding results indicated the impacts of five variables on kojic acid production (g/l) over 41 separate runs: Glucose (A), Yeast extract (B), KH_2PO_4 (C), $MgSO_4.7H_2O$ (D) and pH (E) on kojic acid production (g/l); nevertheless, the most desired concentrations of the variables must be calculated from the three tested values. Derringer's desirability function was used to calculate an optimal variable concentration for high kojic acid production (g/l). By applying the function, the obtained optimum levels were Glucose (150, g/l) (A), Yeast extract (5 g/l) (B), KH_2PO_4 (3 g/l) (C), $MgSO_4.7H_2O$ (0.5 g/l) (D) and pH (3) (E) were found to be the best levels after using the function, which gives desirability equal to 1.000 as cleared at figure (5). In addition, five trials of the optimum parameters were carried out for model validation, and the mean values were compared to anticipated values. The actual values matched the projected values once the desirability functions were applied, demonstrating the sufficiency of the quadratic model devised for increasing production.

Effect of *Aspergillus oryzae* ASU44, biological kojic acid and synthetic kojic acid on cotton aphid

Impact of a fungal strain, its kojic acid extract, and synthetic kojic acid on *A. gossypii* was studied. Every 24 hours, the mortality rate was measured during the treatment period (which lasted 6 days) (Table 3). Aphid mortalities differ significantly at various concentrations and time periods (days). As the concentrations grew, the death rate of *A. gossypii* increased. Also, pathogenicity increased with the time intervals were extended. Table 3 shows the pathogenicity of the fungal kojic acid extract on aphids, *A. gossypii*. On the first day, mortality increased steadily from 0.0±0.0 to 36.0±1.0 at various doses of extract (0, 25, 50, 75 and 100ppm). With a progressive rise in extract concentration, mortality percent ranged from 0.0±0.0 to 52±2.55 on the second day of treatment. Furthermore, varied quantities of extract exhibit a substantial increase in mortality % after 3 days of exposure. Mortality was 0.0±0.0, 38±1.22 and 53±1.22 at 0, 25 and

50 ppm, respectively. At 75 and 100 ppm concentrations of fungal extract, no significant increase in mortality percent was found. At the 4th day after treatment, mortality ranged from 2.0±1.22 to 70±2.74 at various fungal extract concentrations. At varying doses of the extract, mortality climbed steadily from 10.0±1.58 to 87±1.22 at 5th day. The rate of mortality rose with the passage of time, peaking on the sixth day of exposure. At varied concentrations (0, 25, 50, 75, and 100 ppm), the % at the 6th day was 16.0±1.87, 81±1.87, 90±2.24, 95±0.0 and 98±1.22, respectively. There were no significant variations between 75 and 100 ppm values during intervals of days after treatment, according to the findings.

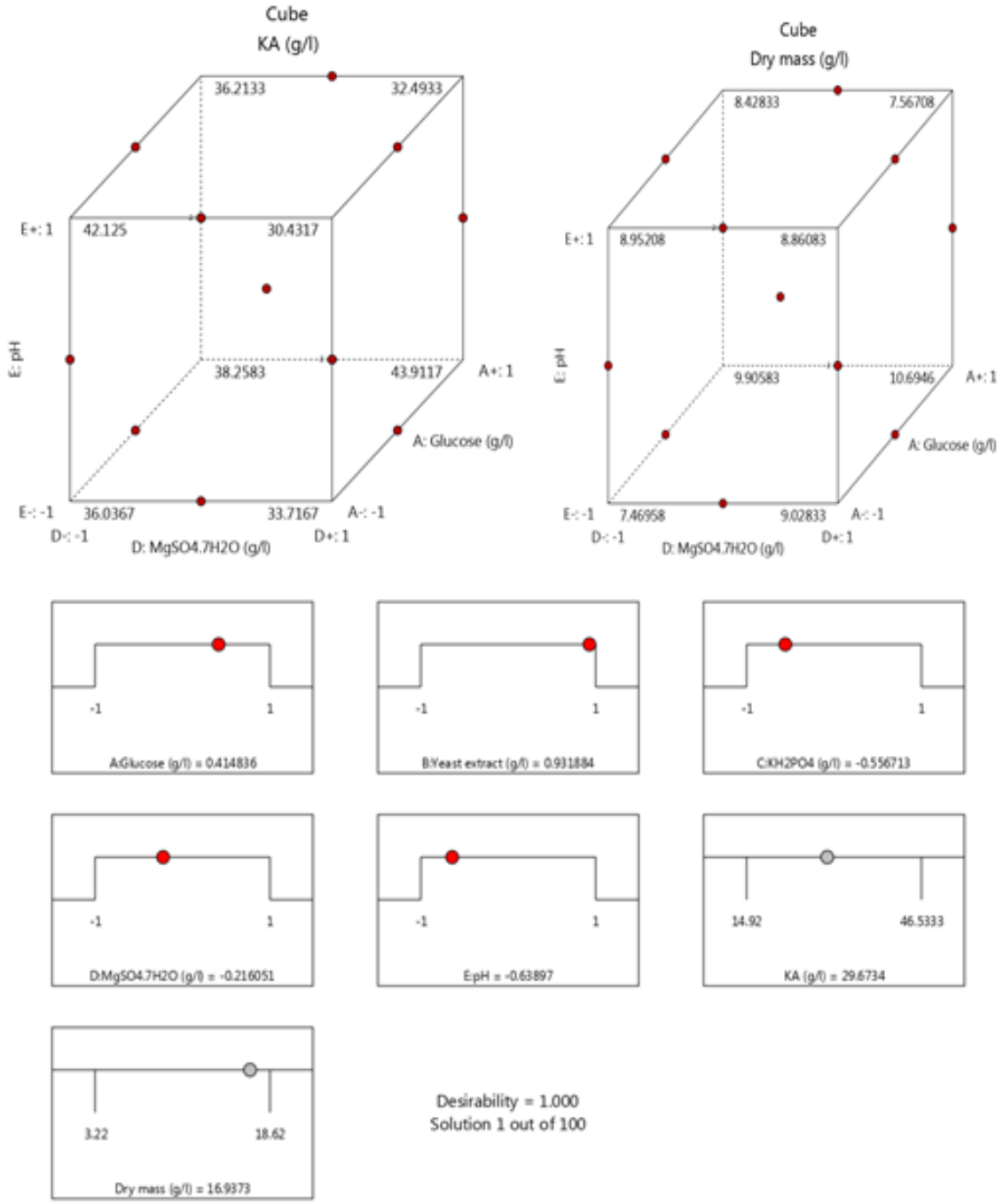

Fig. 5: The statistical optimization desirability ramp plot for kojic acid (g/l) production and dry mass (g/l) by *Aspergillus oryzae* ASU44 (OL314732).

Table 3: Mortality (%) of *A. gossypii* at various concentrations of fungal kojic acid extract of *A. oryzae* ASU44, synthetic kojic acid, and inoculum of *Aspergillus oryzae* ASU44

Fungal kojic acid extract of *A. oryzae* ASU44						
Conc. (ppm)	Days post treatment					
	1day	2days	3days	4days	5days	6days
0.0	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{d}	2.0±1.22^{d}	10.0±1.58^{d}	16.0±1.87^{d}
25	10.0±0.0^{c}	29.0 ±1.0^{c}	38.0±1.22^{c}	49.0±1.0^{c}	60.0±1.58^{c}	81.0±1.87^{c}
50	26.0±1.87^{b}	44.0 ±1.87^{b}	53.0±1.22^{b}	59.0±1.0^{b}	68.0±1.22^{b}	90.0±2.24^{b}
75	34.0±2.45^{a}	46.0±2.45^{b}	57.0±1.22^{a}	68.0±1.22^{a}	83.0±1.22^{a}	95.0±0.0^{a}
100	36.0±1.0^{a}	52.0±2.55^{a}	59.0±1.00^{a}	70.0±2.74^{a}	87.0±1.22^{a}	98.0±1.22^{a}
Synthetic kojic acid						
Conc. (ppm)	Days post treatment					
	1day	2days	3days	4days	5days	6days
0.0	0.0±0.0^{b}	0.0±0.0^{d}	0.0±0.0^{e}	2.0±1.22^{e}	10.0±1.58^{e}	16.0±1.87^{e}
25	0.0±0.0^{b}	0.0±0.0^{d}	12.0±1.22^{d}	18.0±1.22^{d}	28.0±1.22^{d}	33.0±1.22^{d}
50	0.0±0.0^{b}	10.0±0.0^{c}	22.0±1.22^{c}	28.0±1.22^{c}	33.0±1.22^{c}	42.0±1.22^{c}
75	0.0±0.0^{b}	17.0±1.22^{b}	31.0±1.00^{b}	40.0±0.0^{b}	53.0±1.22^{b}	62.0±1.22^{b}
100	5.0±0.00^{a}	20.0±0.0^{a}	40.0±2.24^{a}	53.0±1.22^{a}	64.0±1.00^{a}	72.0±1.22^{a}
Inoculum of *Aspergillus oryzae* ASU44						
Conc. (spores/ml)	Days post treatment					
	1day	2days	3days	4days	5days	6days
0.0	0.0±0.0^{d}	0.0±0.0^{d}	0.0±0.0^{e}	2.0±1.22^{e}	10.0±1.58^{d}	16.0±1.87^{d}
2.5×10^{3}	20.0±0.0^{c}	37.0±1.22^{c}	40.0±0.0^{d}	58.0±1.22^{d}	75.0±2.24^{c}	75.0±2.24^{c}
5×10^{3}	22.0±1.22^{c}	41.0±1.00^{bc}	50.0±0.0^{c}	62.0±1.22^{c}	82.0±1.22^{b}	84.0±1.22^{b}
7.5×10^{3}	26.0±1.00^{b}	46.0±1.87^{ab}	57.0±1.22^{b}	72.0±1.22^{b}	84.0±1.87^{b}	86.0±1.87^{b}
10^{4}	37.0±2.00^{a}	51.0±2.92^{a}	64.0±1.87^{a}	79.0±1.00^{a}	93.0±1.22^{a}	94.0±1.22^{a}

Data are presented as Mean ±SE, n=5. Means followed by the same letter vertically are not significantly different (P≤0.05).

The pathogenicity of *Aspergillus oryzae* ASU44 (OL314732) on *A. gossypii* was examined after 6 days of exposure. Table 3 shows the information revealed appositive substantial increase in aphid mortality over the course of the trial at various doses of fungal spores. At varying concentrations of the fungal inoculum of *A. oryzae* ASU44, on the first day;

remarkable increase in aphid death percentage was noticed. At inoculum concentration of 10^4, mortality jumped to 51±2.92 after the second day of exposure. The death rate gradually increased from 0.0±0.0 to 64±1.87on the third day after exposure. At the fourth day, aphid mortality was 2±1.22, 58±1.22, 62±1.22, 72±1.22 and 79±1.00 at serial inoculum doses of *A. oryzae* ASU44 (0, 2.5×10^3, 5×10^3, 7.5×10^3 and 10^4) correspondingly. The highest effect of this fungal strain was seen on the fifth day after treatment, with a mortality rate of 93±1.22 at the inoculum concentration of 10^4. Mortality rates increased gradually in the remaining inoculum concentration (10±1.58, 75±2.24, 82±1.22 and 84±1.87), respectively. At the final day of exposure, aphid mortality slightly changed and varied from 16.0±1.87 to 94.0 ± 1.22.

The effects of synthetic kojic acid on adult *A. gossypii* were studied and showed in Table3. During six days after treatment, the percentage of aphids that died grew dramatically and steadily with increasing concentrations, according to data analysis. At 25, 50 and 75 ppm of kojic acid, there is no reported mortality on the first day of exposure. This value was 5.0±0.00 at 100 ppm. Then, there was a slightly increase in mortality reached to 20% after 48 hour post treatment. Aphid mortality ranged from 0 to 40.0±2.24 with different levels of kojic acid during 3 days of exposure. At different kojic acid concentrations (0, 25, 50, 75, and 100 ppm), mortality rates were 2±1.22, 18.0±1.22, 28.0±1.22, 40.0±0.0 and 53.0±1.22 on the fourth day after infection. The last two days recorded the highest fatality rates; it ranged from 10.0±1.58 to 64.0±1.00 at different concentrations at 5^{th} days of treatment. Then, aphid mortality values reached to 16, 33, 42, 62 and 72 % at 0, 25, 50, 75 and 100 ppm after 6^{th} days of exposure.

The correct lethal concentration (LC) in controlling *A. gossypii* can be determined *via* probity analysis (Table 4). The LC_{50} value indicated that a concentration of 11.20 ppm of fungal kojic acid extract was required to kill 50% of *A. gossypii*. At a dosage of 50.34 ppm of this extract, cotton aphid could be effectively controlled, with 90 percent mortality. Fungal spores of *A. oryzae* ASU44 at the inoculum concentration 10.94×10^2 (spores/ml) was required to control 50% of aphids. Then, *A. oryzae* ASU44 with inoculum concentration of 9.70×10^3 (spores/ml) was required to kill 90% of *A. gossypii*. The lethal concentration of synthetic kojic acid required to control 50% of the aphid population is 68.18 ppm. Concentration of synthetic kojic acid required for 90% mortality was 274.09 ppm. Data indicated that the required lethal concentration of fungal kojic acid extract was lesser than that of synthetic kojic acid by 10 folds. Lethal time (LT) was calculated and deposited in Table 5. The data showed that as concentration increased, the fatal time values reduced. Remarkably, lowest concentrations were in convenient with highest values of LT. On the other hand, comparing to *A. oryzae* ASU44 and their kojic acid extract, the synthetic kojic acid has the lowest fatal values.

Table 4: Fatal concentrations (LC_{50} and LC_{90}) of *A. oryzae* inoculum, their kojic acid extract and synthetic kojic acid against *A. gossypii* following an exposure experiment.

The used agents	LC_{50} &LC_{90} values		95% Confidence limit	
			Lower	Upper
Fungal kojic acid extract (ppm)	LC_{50}	11.20	5.89	21.28
	LC_{90}	50.34	26.49	95.68
Inoculum of *A. oryzae* ASU44 (spores/ml)	LC_{50}	10.94×10^2	5.04×10^2	2.38×10^3
	LC_{90}	9.7×10^3	4.47×10^3	2.11×10^4
Synthetic kojic acid (ppm)	LC_{50}	68.18	43.94	105.8
	LC_{90}	274.09	176.64	425.31

Table 5: Lethal time (LT_{50} and LT_{90}) of *A. oryzae* inoculum, their kojic acid extract and synthetic kojic acid against *A. gossypii* following an exposure experiment.

Fungal kojic acid extract of *A. oryzae* ASU44 (ppm)				
Conc.	LT_{50} and LT_{90} values (days)		95% Confidence limit	
			Lower	Upper
25	LT_{50}	3.54	2.58	4.58
	LT_{90}	11.80	8.6	16.2
50	LT_{50}	2.40	1.64	3.49
	LT_{90}	10.64	7.3	15.5
75	LT_{50}	1.93	1.37	2.71
	LT_{90}	8.82	5.08	10.06
100	LT_{50}	1.78	1.17	2.7
	LT_{90}	7.15	5.81	13.4
Synthetic kojic acid (ppm)				
25	LT_{50}	11.24	7.66	16.49
	LT_{90}	30.15	20.55	44.22
50	LT_{50}	7.66	5.11	11.47
	LT_{90}	30.98	20.68	46.39
75	LT_{50}	4.76	3.51	6.45
	LT_{90}	14.32	10.56	19.40
100	LT_{50}	3.79	2.89	4.97
	LT_{90}	10.14	7.74	13.28
A. oryzae inoculum (spores/ml)				
2.5×10^3	LT_{50}	2.97	1.95	4.54
	LT_{90}	13.43	8.80	20.50
5×10^3	LT_{50}	2.50	1.70	3.68
	LT_{90}	9.87	6.70	14.53
7.5×10^3	LT_{50}	2.12	1.45	3.10
	LT_{90}	8.02	5.48	11.73
1×10^4	LT_{50}	1.66	1.15	2.41
	LT_{90}	5.97	4.12	8.65

Discussion

Some chemical pesticides are banned in industrialized countries due to their harmful impact on the environment, humans, and other organisms[37]. Alternative microbial control is low-cost, simple to apply, effective, and non-hazardous to humans and the environment. As a result of these advantages, a huge number of innovative fungus-based bio-pesticide products have been commercialized[34]. Furthermore, extracts may contain secondary metabolites or substances with varying insecticidal properties[38]. Kojic acid; a fungal metabolite generated by a variety of fungal species has recently attracted interest due to their antimicrobial and insecticidal qualities[39].

The current research could lead to the creation of a more effective insecticide to combat sap-sucking aphids. The selected bio-control agents were effective in preventing aphid survival in laboratory conditions and resulted in very substantial variations in aphid mortality rates. As a result, the current findings imply that these control bio-agents could be useful in controlling aphid cotton. Previous research has shown that certain fungi are effective against insects, implying that certain fungi contain potent chemicals that might be utilized to control aphids[40]. Toxic to immature milkweed bugs, *Oncopeltus fasciatus* (Dallas), house flies, *Musca domestica* Linnaeus, and a mosquito, *Aedes atropalpus*, *Aspergillus flavus* produced a water-soluble chemical that was eventually identified as kojic acid[21&41].

Using box-Behnken statistical design (41 runs) enhanced kojic acid production from (by) *Aspergillus oryzae* ASU44 (OL314732) to 46.53 g/l (predicted values 46.62 g/l) using Glucose (150, g/l) (A), Yeast extract (5 g/l) (B), KH_2PO_4 (3 g/l) (C), $MgSO_4.7H_2O$ (0.5 g/l) (D) and pH (3). After optimization of kojic acid the researcher reached to varied quantities; Ogawa (1995)[42] obtained 20 g/l kojic acid (KA)

by *A. oryzae* NRRL 484, Wakisaka (1998)[43] produced 24 g/l KA by *A. oryzae*, Futamura (2001)[44] obtained 40 g/l KA by *A. oryzae* MK-107-39, Wan (2005)[45] 41 g/l KA, Yan (20١4)[27] 33 g/l KA, and Liu (2016)[14] obtained 83.47 g/l KA by *A. oryzae*. Our findings imply the insecticidal effects of *A. oryzae* ASU44 culture and its fungal metabolite kojic acid against *A. gossypii* without causing harm to the environment[39]. Similar investigations found that the mycelial extract of *Aspergillus flavus* had mosquito larvicidal action with an LC_{50} value of 34.34 g/ml[40]. Dowd (1988)[22] claimed that fungi extract; kojic acid was poisonous to *Spodoptera frugiperda* and *Hliothis zea*. Trogoderma larvae are inhibited in their growth, and males and females of this species are sterile when exposed to kojic acid[46]. It also stops *M. domestica* and *Drosophila melanogaster* from developing[21].

Kojic acid had a low toxicity, but it slowed insect development and could be insecticidal in large doses, according to Beard and Walton (1969)[21].This is agreeable with our results that indicated that synthetic kojic acid had low effect compared to fungal kojic acid extract. The chosen fungal strain resulted in a mortality rate that was proportional to exposure time and concentrations. Askary (2010)[47], who showed an increase in mortality as time and concentration increased. Kim (2009)[48] found that the concentration or dose of supernatant had a direct relationship with aphid population reduction. Mortality rates may differ due to changes in the strains tested, concentrations, laboratory circumstances, and the material used. In addition, insects reared on food with experimental inoculation of *Aspergillus niger* had a 29% higher mortality rate[41]. This percent mortality is similar to current research, in which *A. gosspii* treated with *A. oryzae* ASU44 demonstrated a gradual increase in death from 20 to 37 percent in the first day when compared to untreated aphids. Also, mortality climbed observed as time passed. With higher doses and treatment periods, kojic acid-related mortality rates increased. According to Alverson (2003)[41], higher doses of kojic acid had a deleterious effect on all aspects of biological fitness. After freshly hatched 1st instar larvae were fed on various mass ratios of kojic acid (0.03, 0.99, 1.66, 2.33, and 2.99 percent) for 7 days, relative mortality increased from 22.2 to 86.7 percent, according to Zhang (2018)[49].

The correct lethal concentration and lethal period in controlling *A. gossypii* can be determined *via* probity analysis. Probity analysis was a method of calculating an insecticide's toxicity against experimental insects. The lethal concentration, LC, can be used to express toxicity levels, the larger the LC value, the more organisms die. Understanding the median fatal dose, lethal concentration, and toxicity is critical for a more accurate assessment of the pesticide's toxicity[50&51]. Data clearly showed that *A. gossypii* was vulnerable to fungal inoculum and their extracted kojic acid. Thus, the used fungus and its kojic acid extract may be useful as effective bio-pesticides against aphids. For spore suspension of *A. oryzae* ASU44, their kojic acid extract, and synthetic kojic acid, the LC_{50} was 10.94×10^2 (spores/ml), 11.20 ppm, and 68.18 ppm, respectively. LC_{90} was 9.70×10^3spores/ml, 50.34 ppm, and 274.09 ppm. Unlike to the present results, the LC_{50} for kojic acid's effect on *Ospheranteria coerulescens* was found to be 23.31[52]. Furthermore, the toxicity value could be expressed as a time limit for death[36&50]. When compared to aphids treated with lesser concentrations of the tested bio-agents, the results demonstrated that aphids treated with high concentrations work faster to control. This suggests that high levels of kojic acid would be able to keep the population of *A. gossypii* under control. The smaller the LT_{50} and LT_{90}, the faster time required to kill *A. gossypii* is decreasing resulting in improved time efficiency[43]. Results indicated that lethal concentration and lethal time values of kojic acid extracted from the *A. oryze* culture is smaller than that of the chemical form of synthetic kojic acid. Consequently, the laboratory prepared fungal kojic acid extract may be more effective in aphid controlling, reducing environment pollution of chemical pesticides.

Conclusions

It concluded that the used of *A. oryze* inoculum and its kojic acid extract were more effective on aphid controlling under laboratory conditions. They exhibit a gradual mortality rates on treated aphids. Moreover, lethal

concentrations and lethal time values indicated that they are effective against aphids. So that, they are suggested to be used in future field studies and could be used as a microbial pesticides in aphid management. In addition, lethal concentration and lethal time of fungal kojic acid extract are lesser than those of synthetic kojic acid indicating that the biological effect was more than the chemical. Thus, little dose of the fungal kojic acid extract can be used in aphid controlling compared to the chemical substrate of kojic acid. Moreover, using of laboratory utilized kojic acid may be easy and cheap method in comparison to its chemical form.

List of Abbreviations

Czapek's dextrose agar (CzDA).Cetyltrimethyl ammonium bromide (*CTAB*). Deoxyribonucleic acid (*DNA). Polymerase chain reaction* (*PCR*) .Internal transcribed spacer (*ITS*) .Thin layer chromatography (TLC). Analysis of variance (ANOVA). Dinitrosalicylic acid (DNS). Statistical Package for the Social Sciences (SPSS).

Availability of data and materials

The following information was supplied regarding data availability: fungal sequencing data are deposited in the NCBI (http://www.ncbi.nlm.nih.gov) web site under accession numbers; *Aspergillus oryzae* ASU44 (OL314732).

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Authors' contributions

GAM; participate in the experiment design, data analysis, and manuscript writing, AAZ; supervision and manuscript revision, NAK; participate in the practical work of the mycological part, NMRA; participate in the practical work and writing of insect part. The final manuscript approved by all authors.

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إستخدام فطرة *Aspergillus oryzae* ASU44 (OL314732) وحامض الكوجيك المنتج بواسطتها كمبيدات للآفات ضد حشرة مَن القطن

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بسبب التأثيرات السلبية على البيئة والانسان والكائنات الأخرى تم حظر مبيدات الآفات المصنعة كيميائيا في العديد من الدول. وقد وجد ان المكافحة الميكروبية لتلك الآفات تتميز بأنها ذات فاعلية، سهلة الأستخدام واكثر اماناً علي الانسان والبيئة. هذا والمبيدات البيولوجية المنتجة بواسطة الفطريات مثل حامض الكوجيك يمكن إنتاجها تجارياً كبديل فعال للمبيدات الكيميائية. فطرة Aspergillus oryzae ASU44 (OL314732) تم عزلها من المنطقة المحيطة بجذور نبات الخطمية وتم تعريفها جينياً باستخدام S rRNA١٨ وتبين ان تلك السلالة لها قدرة عالية علي انتاج حامض الكوجيك وخاصة بعد تحسين الإنتاج باستخدام التصميم الاحصائي Box-Behnken بـ ٤١ تجربة وكانت أعلي كمية تم الحصول عليها من حامض الكوجيك هي ٤٦,٥٣ جم/ لتر من تجربة رقم (٢٣) باستخدام جلوكوز ١٥٠جم/لتر، مستخلص خميرة ٥جم/لتر، ثنائي فوسفات البوتاسيوم ٣جم/لتر، كبريتات الماغنيسيوم المائية ٠,٥جم/لتر، pH 3 وتم اختبار تأثير الفطر وحامض الكوجيك المنتج بواسطة الفطر والمنتج كيميائياً علي مَن القطن وكان لهم تأثير علي معدل الوفيات للحشرة. وكان معدل الوفيات مرتبط بكمية وفترة التعرض لهذه المبيدات البيولوجية وأظهرت النتائج انه كلما زاد التركيز كلما قل الوقت اللازم لقتل الحشرة وقد تبين ان الفطر وحامض الكوجيك المنتج منه لهما تأثير فعال علي مَن القطن ويمكن استخدامهما في مكافحة تلك الآفة الضارة بمحصول القطن.