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Compatibility of entomopathogenic fungi and *Azadirachta indica* extract against the cotton pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) under controlled conditions

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Abstract

Compatibility of entomopathogenic fungi (EPFs) viz. *Verticillium lecanii*, *Metarhizium anisopliae* and *Beauveria bassiana* with *Azadirachta indica* extract (alone and in combinations) was evaluated against 2nd instar larvae of the cotton pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) under controlled conditions. Results indicated that maximum mortality rate of *P. gossypiella* larvae was recorded at combination of the highest conidial concentrations of *V. lecanii* + *M. anisopliae* + *B. bassiana* along with *A. indica* extract, while minimum mortality rate was recorded at *A. indica* extract alone, 12 days post exposure interval. Maximum mycosis and sporulation from dead cadavers of *P. gossypiella* larvae were recorded at the highest concentration of *B. bassiana*, while the lowest mycosis and sporulation were recorded at the highest conidial concentrations of *V. lecanii* + *M. anisopliae* + *B. bassiana* along with *A. indica* extract. Maximum percent of pupation and adult emergence in *P. gossypiella* larvae was observed in the treatment, where *A. indica* extract was applied alone, while the minimum percent of pupation and adult emergence was observed at the treatment combination of the highest concentration of *B. bassiana* + *V. lecanii* + *M. anisopliae* along with *A. indica* extract was applied. EPFs proved their enhanced long-term protection potential for cotton crop against *P. gossypiella*. *A. indica* extract integrated with the EPFs can be a potential alternative to the chemicals and as an effective component of IPM program against *P. gossypiella*.

Keywords: Cotton pink bollworm, *Pectinophora gossypiella*, Entomopathogenic fungi, *Azadirachta indica*, Virulence, Biological control

Background

The cotton pink bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae), is one of the major economic cotton pests that causes considerable damages

to cotton in Pakistan (Jaleel et al. 2014) and many other countries (Parmar and Patel 2016). Different approaches such as chemical insecticides and growing resistant cultivar (transgenic *Bt* cotton containing Cry1Ac toxin) have been used to manage the pest control (Heuberger et al. 2014), but they have not given optimal control levels of the pest (Mohamed et al. 2016). Plant extracts such as *Nicotiana tabacum* and *Azadirachta indica* have widely

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been used to control insect pests. *A. indica* (Neem) has been used for years in Indo-Pak against several insect pests and is still used for stored grain pest (Rajendran and Sriranjini 2008). Due to its broad host range, inexpensive production and no harmful impact on environment (Mathew 2016) makes it a safer alternative method to control some insect pests.

The entomopathogenic fungi (EPFs) are among the most effective and environmental friendly biological control agents that invade their host insect through the cuticle and play a key role in the regulation of insect pest population in natural ecosystem (Niu et al. 2019). EPFs can be used against a wide range of insect pests and their nonspecific actions and antagonistic natures give them broad host range ability (Ong and Vandermeer 2014). More than 700 species of fungi belonging to 90 genera among *Beauveria bassiana*, *Metarhizium anisopliae*, *Verticillium lecanii*, *Purpureocillium lilacinum*, and *Isaria fumosorosea* are the widely used ones as biological control agent against many agricultural pests (Khan et al. 2012; Rizwan et al. 2019). The addition of plant extracts which act as both adjuvant (Nursal and Ilyas 2019) and bio-pesticide (Dougoud et al. 2019) can heighten the coverage of leaf and persistence of EPFs (Świergiel et al. 2016) resulting in enhanced performance of EPFs and plant extracts in combination for the suppression of some insect pests such as *P. gossypiella* (Vashisth et al. 2019).

This study aimed to check the effect of EPFs; *B. bassiana*, *M. anisopliae* and *V. lecanii* and the plant extract (*Azadirachta indica*) on some biological aspects of *P. gossypiella* under laboratory conditions.

Materials and methods

P. gossypiella culture

Different growth stages of *P. gossypiella* larvae were collected from cotton fields where their population did not expose to any insecticidal applications. All the stages of *P. gossypiella* were placed separately in labeled plastic vials and transferred to the laboratory. Larvae were maintained by feeding them on green bolls at $27 \pm 0.5^\circ\text{C}$ until pupation. Larval discrepancy on the basis of their sex was performed, following the method of Dharajothi et al. (2010) for moth copulation (Jothi et al. 2016). Moths were released in insect rearing cages measuring 28 cm height and 24 cm diameter for egg laying, at the rate of 20 pairs per cage, and were fed upon 1 ml multivitamin and protein mixed with 100 ml honey solution (20%) along with fresh terminal buds and leaves of cotton brought from unsprayed cotton plot, inserted in a small conical flask contains water to keep them fresh egg lying space for adults (Muralimohan et al. 2009). Water-soaked cotton twigs were placed at the bottom of the flask to maintain the moisture level of the tissues and were replaced every 2nd day. These twigs were

transferred into translucent plastic containers (5 cm height and 4 cm diameter) sealed with muslin cloth and rubber band for egg hatching (Parker 2005).

The larvae were maintained in plastic trays on green bolls at $26 \pm 2^\circ\text{C}$, $65 \pm 5\%$ R.H. and 11 h light–13 h dark photoperiod until pupation. The pupae were placed in plastic vials (4 × 5 cm diameter × height) with a mesh-windowed lid and a disk of filter paper at the bottom, retained until adult emergence (Muralimohan et al. 2009). Glass wares were rinsed with distilled water followed by sterilization and were used for the preparation and storing of diet.

Entomopathogenic fungi

Two commercial formulations of 3 EPFs viz. *V. lecanii*, *M. anisopliae* and *B. bassiana* were procured from AgriLife SOM Phytopharma (India) Limited® (www.agrilife.in) in the form of talc powder. Formulations, at 2 different concentrations (1×10^6 and 1×10^8 CFU/ml), were tested against *P. gossypiella*. Hemocytometer and potato dextrose agar (PDA) were used to determine the conidial concentrations and germination in conidial suspension, respectively. Measurement of conidial germination was computed by randomly counting 200 conidia in each plate at $25 \pm 2^\circ\text{C}$, 18 h after incubation (Atta et al. 2020).

Preparation of conidial suspensions

EPF's conidial suspensions concentrations, 1×10^6 conidia ml⁻¹ and 1×10^8 conidia ml⁻¹ alone and in combination with *A. indica* extract were prepared by dissolving in distilled water and in 5% extract as basic solution while using hemocytometer.

Preparation of *Azadirachta indica* plant extract

Plant extract of *A. indica* was prepared by adopting the methodology of Ali et al. (2017). Fresh collected leaves of *A. indica* were sufficiently washed by distilled water and dried in shadow, followed by electric grinding to get fine powder. Fine *A. indica* powder (50 g) was dissolved in distilled water (500 ml) in a 2.5 liter sized conical flask by heating the solution at 60°C and shaking the flask continuously with a magnetic stirrer for 6 h. Solution was filtered, using Whatman no. 1 after sieving with muslin cloth to remove any solid particles. Rotary evaporator was used to evaporate the solution in vacuumed conditions in hot air oven at to bring the dry plant extract to a constant volume 50 ml. The solution thus obtained was considered as 100% *A. indica* extract, stored at 4°C for further investigations.

Bioassay

Thirty fresh molted 2nd instar *P. gossypiella* larvae of uniform brood were treated by immersing them in to the conidial suspension (1×10^6 and 1×10^8 ml⁻¹)

concentrations, i.e., *B. bassiana*, *V. lecanii*, *M. anisopliae* and *A. indica* extract (5%) alone and in combination for 10 s (Derbalah et al. 2014). After the treatment, larvae were placed into sterile Petri dishes (9-cm diameter) for air drying for 10 min. The treated larvae were maintained in labeled plastic trays with artificial diet for further investigations, i.e., mortality, sporulation, mycosis, pupation and adult emergence under laboratory conditions. Mortality rate was calculated at 4, 8 and 12 days of time intervals after which sporulation and mycosis were computed. Mortality data was calculated by Abbott's formula (Abbott 1925).

$$\text{Corrected mortality\%} = \left(1 - \frac{n \text{ in } T \text{ after treatment}}{n \text{ in } C_0 \text{ after treatment}} \right) \times 100$$

where n = insect population, T = treated, C_0 = control

Sporulation and mycosis

Dead cadavers of *P. gossypiella* stiffs were collected from treatments where EPFs were applied (alone and in combination), for sporulation and mycosis and were transferred to plastic vials from sterile Petri dishes for refrigeration at 4 °C. Solution of sodium hypochlorite (0.05%) was used for surface sterilization of the collected cadavers for 2–3 min, followed by 2–3 washings, using distilled water (Leland and Gore 2016). The cadavers were then placed in the Petri dishes for a week with PDA for incubation at 75 ± 5% R.H. and 25 ± 1 °C. Microscope was used for the observation and identification of external growth of the fungi on the treated cadavers. A drop of Tween-80 was added and stirred for 10 min with distilled water (20 ml) to mix with the cadavers, selected from each replication, which were already mycosed for the determination of sporulation. Hemocytometer coupled with microscope was used to determine the total number of conidia ml⁻¹ (Rizwan et al. 2019).

Assessment of pupation and adult emergence of *P. gossypiella*

All the remained larvae after treatments were evaluated further reared in plastic trays (5.5 cm × 6 cm, depth and diameter) with artificial untreated diet (Muralimohan et al. 2009) to allow them to continue their development until pupal stage to calculate percent pupation. Pupae were placed individually in a Petri dish (8-cm diameter) until adults emerged to calculate percent adult emergence.

Statistical analysis

The data was analyzed with the Statistix® (Version 8.1) statistical package, using analysis of variance (ANOVA) in CRD to determine the effects of individual and

interacted application of variables. Tukey's HSD test for mean separation was used to compare mean values at $P < 0.05$ (Sokal and Rohlf 1995).

Results and discussion

Mortality rates of *P. gossypiella*

Thirty 2nd instar larvae of *P. gossypiella*, treated with different EPFs concentrations and *A. indica* (*AI*) (alone and/or in combination) showed significant effects at different exposure intervals. At 4 and 8 day intervals, all 3 EPFs combined with *AI* extract (*Ba2* + *VI2* + *Ma2* + *AI*) showed the highest mortality of 48.67 and 57.33%, while lowest the mortality rate, recorded in *Bb1* (22.27 and 28.93%), respectively. Similarly at 8 day interval, all the 3 EPFs combined with *AI* extract (*Ba2* + *VI2* + *Ma2* + *AI*) showed the highest mortality rate (74.67%), whereas the lowest one (37.33%) was recorded in *VI1*. Mortality rates of all treatments was compared to the control, where 0.00, 1.33 and 2.67% rates were recorded at 4, 8 and 12 day intervals, respectively (Table 1).

Mycosis and sporulation from dead cadavers of *P. gossypiella*

Effects of the lowest and the highest concentrations of EPFs, alone and in combination, on percent mycosis and sporulation (conidia ml⁻¹) from dead cadavers of treated 2nd instar *P. gossypiella* larvae were recorded highly significant ($P < 0.01$) (percent mycosis: $F_{6,34} = 46.7$; sporulation: $F_{6,34} = 48.7$). Maximum percent mycosis and sporulation (94.20 ± 1.10% and 157.20 ± 1.67 conidia ml⁻¹, respectively) was recorded at the dead cadavers of *B. bassiana* (*Bb2*)-treated *P. gossypiella* at the highest concentration (1×10^8 conidia ml⁻¹), while the minimum percent mycosis and sporulation (37.20 ± 1.31% and 103.40 ± 1.05 conidia ml⁻¹, respectively) was recorded at the dead cadavers of *P. gossypiella* treated with combination of *A. indica* + the highest concentration (1×10^8 conidia ml⁻¹) of *B. bassiana*, *V. lecanii* and *M. anisopliae* (*Bb2* + *VI2* + *Ma2* + *AI*) (Table 2). These results indicated that *AI* demonstrated inhibitory effects on the mycosis and sporulation.

Pupation and adult emergence from treated second larval instar of *P. gossypiella*

Effects of both lowest and the highest concentrations of EPFs, alone and in combination with *A. indica* extract, while that of the highest concentration of EPF (1×10^8 conidia ml⁻¹) on percent pupation and progeny of the 2nd instar *P. gossypiella* larvae, were highly significant ($P < 0.01$) (percent pupation: $F_{11,59} = 37.0$; percent adult emergence: $F_{11,59} = 22.8$). Maximum percent pupation and adult emergence (90.67 ± 1.28% and 84.00 ± 2.47%, respectively) from *P. gossypiella* were recorded in control, while minimum percent pupation

Table 1 Percent mortality (mean ± SE, n = 5) of 2nd instar *Pectinophora gossypiella* larvae due to *Beauveria bassiana*, *Verticillium lecanii*, *Metarhizium anisopliae* concentrations and *Azadirachta indica* extract (alone and in combination) at different exposure intervals. Means sharing with same lower case letters are not significantly different from each other at 0.05 level of significance, i.e., P < 0.05

Treatments	4 days	8 days	12 days
Bb1	22.27 ± 1.75p	28.93 ± 1.23nop	44.80 ± 2.04hi
Bb2	26.27 ± 2.24op	34.13 ± 2.14l	53.60 ± 1.95de
Vl1	24.67 ± 2.03p	31.33 ± 1.61mn	37.33 ± 2.08jk
Vl2	24.67 ± 1.98p	34.67 ± 2.43l	44.67 ± 2.24hi
Ma1	27.33 ± 1.73op	37.33 ± 1.91jk	46.67 ± 2.11fgh
Ma2	31.33 ± 2.03mn	39.33 ± 2.13jk	50.67 ± 2.76def
AI	23.33 ± 1.05p	30.67 ± 1.59nop	40.67 ± 2.23ij
Bb2 + AI	34.67 ± 1.21l	44.67 ± 1.92hi	60.67 ± 2.64c
Vl2 + AI	37.33 ± 1.91jk	48.67 ± 1.86fg	64.67 ± 1.80bc
Ma2 + AI	40.67 ± 3.38ij	54.67 ± 2.03de	66.67 ± 2.11b
Bb2 + Vl2 + Ma2 + AI	48.67 ± 2.29fg	57.33 ± 2.80cd	74.67 ± 2.43a
Control	0.00 ± 0.00q	1.33 ± 0.37q	2.67 ± 0.56q

Bb1 *Beauveria bassiana* (1 × 10⁶ conidia ml⁻¹), Bb2 *Beauveria bassiana* (1 × 10⁸ conidia ml⁻¹), Vl1 *Verticillium lecanii* (1 × 10⁶ conidia ml⁻¹), Vl2 *Verticillium lecanii* (1 × 10⁸ conidia ml⁻¹), Ma1 *Metarhizium anisopliae* (1 × 10⁶ conidia ml⁻¹), Ma2 *Metarhizium anisopliae* (1 × 10⁸ conidia ml⁻¹), AI *Azadirachta indica* extract

and adult emergence were zero recorded in *P. gossypiella* treated with a combination of the highest concentration of *B. bassiana* + *V. lecanii* + *M. anisopliae* (1 × 10⁸ conidia ml⁻¹) and *A. indica* (Bb2 + Vl2 + Ma2 + AI). Alone *A. indica* extract-treated *P. gossypiella* demonstrated 87.33 ± 1.73% pupation and 64.67 ± 3.08% adult emergence (Table 3).

Table 2 Percent mycosis and sporulation (conidia ml⁻¹) (mean ± SE, n = 5) from dead cadavers of 2nd instar *Pectinophora gossypiella* larvae due to the highest concentrations of *Beauveria bassiana*, *Verticillium lecanii*, *Metarhizium anisopliae* (1 × 10⁸ conidia ml⁻¹) (alone and in combination). Means sharing with same lower case letters are not significantly different from each other at 0.05 level of significance, i.e., P < 0.05

Treatments	Percent mycosis	Sporulation (conidia ml ⁻¹)
Bb1	87.40 ± 1.06a	147.40 ± 1.04ab
Bb2	94.20 ± 1.67a	157.20 ± 1.10a
Bb2 + AI	51.20 ± 1.45cd	126.40 ± 1.17def
Vl1	82.20 ± 1.55a	142.20 ± 1.24bc
Vl2	87.20 ± 1.30a	146.40 ± 1.05abc
Vl2 + AI	43.20 ± 0.86de	117.20 ± 1.05f
Ma1	61.40 ± 1.20bc	129.20 ± 0.88de
Ma2	65.20 ± 1.24b	135.20 ± 1.15cd
Ma2 + AI	48.40 ± 1.06de	123.20 ± 1.24ef
Bb2 + Vl2 + Ma2 + AI	37.20 ± 1.05e	103.40 ± 1.31g
Control	0.00 ± 0.00f	0.00 ± 0.00h

Bb1 *Beauveria bassiana* (1 × 10⁶ conidia ml⁻¹), Bb2 *Beauveria bassiana* (1 × 10⁸ conidia ml⁻¹), Vl1 *Verticillium lecanii* (1 × 10⁶ conidia ml⁻¹), Vl2 *Verticillium lecanii* (1 × 10⁸ conidia ml⁻¹), Ma1 *Metarhizium anisopliae* (1 × 10⁶ conidia ml⁻¹), Ma2 *Metarhizium anisopliae* (1 × 10⁸ conidia ml⁻¹), AI *Azadirachta indica* extract

The EPFs conidial suspension concentrations alone, or combined with, *A. indica* extract showed promising potentials against *P. gossypiella* larvae by causing significantly high mortality rates after treatment within 4, 8 and 12 day intervals. *B. bassiana*, *V. lecanii* and *M. anisopliae* concentrations and *A. indica* extract (alone and in combination) at different exposure intervals showed

Table 3 Percent pupation and percent adult emergence (mean ± SE, n = 5) of 2nd instar *Pectinophora gossypiella* larvae due to *Beauveria bassiana*, *Verticillium lecanii*, *Metarhizium anisopliae* concentrations and *Azadirachta indica* extract (alone and in combination). Means sharing with same lower case letters are not significantly different from each other at 0.05 level of significance, i.e., P < 0.05

Treatments	Percent pupation	Percent adult emergence
Bb1	80.67 ± 2.08ab	57.33 ± 2.80bc
Bb2	77.33 ± 2.18abc	54.67 ± 2.24bcd
Vl1	64.67 ± 2.61cd	41.33 ± 2.24de
Vl2	74.00 ± 3.11bc	47.33 ± 2.88cde
Ma1	54.00 ± 3.03d	32.67 ± 2.34ef
Ma2	57.33 ± 3.87d	34.00 ± 3.25e
AI	87.33 ± 1.73ab	64.67 ± 3.08b
Bb2 + AI	27.33 ± 1.73e	18.00 ± 2.34fg
Vl2 + AI	14.00 ± 1.28ef	7.33 ± 0.87gh
Ma2 + AI	24.67 ± 1.38e	14.67 ± 1.38gh
Bb2 + Vl2 + Ma2 + AI	0.00 ± 0.00f	0.00 ± 0.00h
Control	90.67 ± 1.28a	84.00 ± 2.47a

Bb1 *Beauveria bassiana* (1 × 10⁶ conidia ml⁻¹), Bb2 *Beauveria bassiana* (1 × 10⁸ conidia ml⁻¹), Vl1 *Verticillium lecanii* (1 × 10⁶ conidia ml⁻¹), Vl2 *Verticillium lecanii* (1 × 10⁸ conidia ml⁻¹), Ma1 *Metarhizium anisopliae* (1 × 10⁶ conidia ml⁻¹), Ma2 *Metarhizium anisopliae* (1 × 10⁸ conidia ml⁻¹), AI *Azadirachta indica* extract

the highest mortality rates. Similarly, at 8 day intervals, all the 3 EPFs combined with *AI* extract (*Ma2* + *Vl2* + *Ma2* + *AI*) showed the highest mortality rate (74.67%). In the present investigations, *P. gossypiella* pupation as well as adult emergence were also significantly affected. *B. bassiana*, *V. lecanii* and *M. anisopliae* concentrations and *A. indica* extract (alone and in combination) at different exposure intervals caused high mortality rates among the treated 2nd instar *P. gossypiella* larvae due to their effects on specific hydrolytic enzyme such as proteinase, chitinase and lipase that affect cuticle (Kurtti and Keyhani 2008).

Introduction of an exogenous biological agent into an environment, with the aim towards its permanent establishment to control the pests present therein has been an effective technique over the long term (Kenis et al. 2017). Maximum percent mycosis and sporulation was recorded from the dead cadavers of *B. bassiana*-treated *P. gossypiella* at 1×10^8 conidia ml⁻¹ concentration, while minimum percent mycosis and sporulation was recorded from the dead cadavers of combination of the highest concentrations of *B. bassiana* + *V. lecanii* + *M. anisopliae*. The results are in line with the findings of Riasat et al. (2011) who reported that the maximum mycosis (86.47%) and sporulation (153.22 conidia ml⁻¹) were observed in treatments where the lowest concentration of *B. bassiana* (2.23×10^7 conidia Kg⁻¹) alone was applied against adults of *Rhizopertha dominica* (F.) (Coleoptera: Bostrichidae). They also witness our results showing the antagonistic behavior of combined EPFs. They reported low rates of mycosis and sporulation in the treatments, where high concentrations of diatomaceous earth were mixed with *B. bassiana*. Similar results were also documented by Tefera and Pringle (2003), who found the highest mycosis and sporulation in cadavers of *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) treated with *B. bassiana* alone. The results of present finding are in line with the findings of Ashraf et al. (2017) applied 1.4×10^4 ml⁻¹ conidial concentration of *M. anisopliae* individually against stored grain insect pests and reported that percent mycosis and sporulation was high.

Maximum percent pupation and adult emergence in 2nd instar larvae of *P. gossypiella* was observed in treatment where *AI* extract was applied alone, while minimum percent pupation and adult emergence was observed in treatment where combination of the highest concentrations of *B. bassiana* + *V. lecanii* + *M. anisopliae* along with *AI* extract was applied. These results are in agreement with Sufyan et al. (2019), who documented that both pupation and adult emergence of 2nd and 4th larval instars of *C. partellus* were maximum at low concentration of entomopathogens (alone and in combination), while minimum pupation and adult

emergence were recorded at high concentrations of entomopathogens (alone and in combination).

Conclusion

This study concluded that the integration of EPFs and *A. indica* extract can prove a successful alternative to traditional chemicals and may become effective component of IPM program against *P. gossypiella* and some other insect pests. Further studies under field conditions are required.

Abbreviations

EPF: Entomopathogenic fungi; PDA: Potato dextrose agar; *n*: Insect population; *T*: Treated; *Co*: Control; ANOVA: Analysis of variance; CRD: Complete randomized design; HSD test: Honest significance test; *Bb1*: *Beauveria bassiana* (1×10^6 conidia ml⁻¹); *Bb2*: *Beauveria bassiana* (1×10^8 conidia ml⁻¹); *Vl1*: *Verticillium lecanii* (1×10^6 conidia ml⁻¹); *Vl2*: *Verticillium lecanii* (1×10^8 conidia ml⁻¹); *Ma1*: *Metarhizium anisopliae* (1×10^6 conidia ml⁻¹); *Ma2*: *Metarhizium anisopliae* (1×10^8 conidia ml⁻¹); *AI*: *Azadirachta indica* extract

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Authors' contributions

MAF, BA and MDG designed and conducted the experiment, collected and analyzed the data, and wrote manuscript. BA helped in apprehending the idea of this research, designing the layout of experiment and improving the write-up, format, and language of this manuscript. MJA and QAA reviewed the manuscript, add and improved declaration section, edited the format of the tables according to the format of this journal, contributed in data setting for analysis, reviewed the final manuscript, and made the format of this manuscript according to the format of this journal. This final manuscript was ultimately perused, scrutinized, and approved for final submission by all the authors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The authors agree to all the concerned regulations.

Consent for publication

The authors agree to publish this scientific paper at the Egyptian Journal of Biological Pest Control.

Competing interests

The authors declare that they have no competing interest.

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