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Endophytic Bacteria as Apotential Agent for Control of Tomato Wilt Caused by *Fusariumoxysporum F.Sp Lycopersici*

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Abstract

This study was conducted at the college of Agriculture / University of Basrah during 2020/2021 season ,with aim of isolating the endophytic bacteria from internal tissue of healthy tomato plants and their associated weeds and evaluating their efficiency in reducing tomato wilt caused by *Fusarium oxysporum f.sp lycopersici* . Ten isolates out of eighty-two isolate obtained from tomato plants and some associated weeds showed efficiency in inhibiting the growth of the F.O.L both in well and dual culture technique. Identification of endophytic bacteria by VITEK2 device showed that three of them belong to *Pseudomonas aeruginosa* , one belong to *P.putida* while the others belong *Staphylococcus lentus* ,*Enterobacter dissolvens* , *Alloiococcus otitis* and *Serratia marcesens*. Pot experiment showed that soaking tomato seedling roots with suspension of *P.aeruginosa* or *P.putida* led to a reduction of tomato wilt incidence from 100% in control treatment to 41.7% and disease severity from 45.8% to 16.7%,and increased chlorophyll content and polyphenol oxidase and peroxidase activety.

Keyword: Endophyte bacteria, Tomato wilt, control

Introduction:-

Basrah Governorate is one of the main areas in the production of Tomato crop in Iraq specially in winter season. Tomato plant facing many problems including infecting the crop with several insect pests and plant pathogens that affect the quantity and quality of the crop (Al -Athowri, 2002).The tomato crop is affected by several important economic diseases such as early blight, Tomato leaf curl virus and tomato wilt caused by*Fusarium oxysporum f.sp lycopersici*which is consider as the most important diseases of this crop, as the rate of infection in desert farms in Basra is estimated at 22-27%.(Al-Athowri and Fayyadh 2002).Several strategies were used in the management of Fusarium wilt disease, most of which relied on the use of fungicides, agricultural rotation or resistant varieties, etc. However, most of them did not achieve desired results in reducing the losses caused by this fungus due to its ability to infect vascular tissues, which makes it difficult for pesticides to reach it, presence of several strains of the fungus differ in their infectivity to

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different varieties, in addition to the ability of the fungus to remain in the soil for several years in the form of chlamydospores that are resistant to unfavorable conditions, (Fayyad and Abbas, 2019).Biological control is the first strategic line in plant disease management, as it is a safe method for the environment and does not affect non-target organisms, in addition to being one of the basic elements in a sustainable agriculture system (Ali *et al*., 2020). Several biological agents have been used to control plant diseases, such as *Trichoderma spp*., *Pseudomonas spp*. and *Streptomyces spp*. (Fayyad and Abbas, 2019;Awad and Fayyadh .,2018). In recent years, microorganisms that coexist within plants, including the well-known endophytic bacteria, have received increased attention for their benefits in increasing plant tolerance to environmental stress factors in addition to their contribution in protecting plants from disease infections. (White *et al*., 2019).In view of the economic importance of Fusarium wilt disease and the need to search for alternative means for fungicides, this research were done with the aim of evaluating the efficiency of bacteria isolated from the internal tissues of tomato plants and their associatedweeds in reducing the incidence of Fusarium wilt disease.

Materials and methods: -

1- Isolation of the fungus*Fusarium oxysporum f.sp lycopersici.*

Tomato plants showing signs of yellowing and wilting, planted in plastic houses at the College of Agriculture / University of Basra, were collected .The roots of the plants were washed well with tap water and then the roots and the lower part of the stem were cut into small pieces (1.5-1 cm).) , sterilized with sodium hypochlorite solution(NaOCl) at a concentration of 1% free chlorine for three minutes, then washed with sterile distilled water and desiccate on to sterile filter paper.Three pieces were transferred to Petri dishes with a diameter of 9 cm containing sterile PDA medium amended with the antibiotic Chloramphenicol at a rate of 250 mg per liter of culture medium. The plates were incubated at a temperature of 28 ± 2 ° C for 7 days, and were purified according to the single spore technique .the fungus identified according to (Leslie and Summerell, 2006). Pathogenicity of the fungus was tested according to the method of (Bolkan and Bulter., 1974).

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2- Isolation of endophyte bacteria from the tissues of tomato plants and the associated weeds.

Samples were collected from healthy tomato plants(*Lycopersicum esculintum*) and from some of the associated weeds from Al-Zubair, Safwan, Al-Deir and the greenhouses of the College of Agriculture university of Basra. The weeds samples included *Chenopodium album*, *Malva parvifllora*, *Amaranthus biitoides* and the *Melilotus indicus*.. The samples were washed with tap water. The plant parts were divided into leaves, stalk and root. Leaves were cut into small pieces (1- 1.5 cm),sterilized for two interval times with 70% ethyl alcohol for three minutes and washed with sterile distilled water . Then they were desicate and all 4 pieces were transferred by sterile forceps to petri dishes containing the Nutrient agar mediaamended with anti-fungal Nystatin . The dishes were incubated at 30 ° C for 3 days (Hassan *et al*., 2019). On the other hand the stem and roots parts were cut into small pieces (1 - 1.5 cm),then the epidermis has been peeled offby a sterile scalpel to reach the inner tissue, sterilized with NaOCl solution at a concentration of 1% free chlorine for 3 minutes and washed with sterile distilled water and dried with filter paper. four pieces were transferred to Petri dishes containing the nutrient agar (N.A)and placed in the incubator at 30 ° C for 3 days (Zhoa *et al*., 2011), the bacterial isolates were purified by a series of dilution ..

3- Preliminary test for antagonistic activity of endophyte bacteriaagainst*Fusariumoxysporum f.sp lycopersici.*

In this experiment 82 isolates of endophyte bacteria which were isolated from tomato plants and their associated weeds were used, The antagonistic ability of endophyte bacterial isolate was done by agar well technique. 0.5 cm mycelium plug from four-day age PDA culture of *F.oxysporum f.sp lycopersici* were placed in the central well . The four well around the central well were inoculated with a disc of 0.5 cm in diameter for each isolate of pure culture of endophyte bacteria at 48 hours of age and at a distance of 3 cm from the center of the plate. Three replications of each isolate were done, the control treatment included placing disc of sterile culture medium (N.A) in the wellsinstead of endophytebacteria. The plates were incubated at 30 ° C for five days (Nandhini *et al*., 2012). The antagonistic activity was calculated according to following formula (Khamna *etal*., 2009):-

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 3119 – 3132
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C = A - B

C = zone of inhibition.

A = The distance between the fungus disc and the bacterial disc.

B = the growth distance of the fungus towards the bacterium.

This experiment was repeated by dual culture technique ,and the percentage of growth inhibition was calculated according to following formula:-

% of growth inhibition= $\frac{A-B}{A}$ X 100

A=The growth of fungus in control treatment . B=The growth of the fungus in dual treatments.

4- Identification of endophyte bacteria with the VITEK2 device.

The isolates of bacteria were grown on N.A medium, at the age of 24 hours, the isolates were sent to the Al-Bayan laboratory for diagnosing microorganisms, Al-Ashar-Basra to perform the identify of bacterial isolates using the VITEK 2 Compact system produced by the French company BioMerieux. The identification on the bacterial isolates was done according to the method (Funke *etal*.,1998).

5-Effect of some endophytic bacterial isolates on tomato wiltcaused by*F.oxysporum f.sp lycopersici*.

A mixture of soil and peat moss in a ratio of 1: 3 was sterilized with a commercial formalin (40%) in concentration of 2%.Sterilized plastic pots size 22 x 22 cm were filled with three kg of sterilized soil mixture , after that 5 gm of fungus inoculum grown on millet seeds were added for each pot. Roots of tomato seedlings(Supermarmound varity) at the age of three weeks were dipped roots for 20 minutes in suspension of endophytic bacterial isolates prepared in a Nutrient broth medium.Then three seedlings were planted in each pots, and each pot was watered at a rate of 100 ml of bacterial suspension. The experiment was carried out using four isolates of bacteria: *Pseudomonas*. *aeruginosa*.4, *P.aeruginosa.5*, *P. aeruginosa.6* and *P.putida.* 12. Which gave the best results in the inhibition of the pathogenic fungus and identified by VITEC technique.. The experiment was carried out according to a complete random design with four replicate. The control

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treatment included planting seedlings in soil infested with the pathogen fungusonly (control 1) or planting tomato seedling in sterile soil(control 2), for each control treatments 100 ml of N.A was added.At the end of the experiment the following parameters were measured-:Disease incidence,disease severity, and total Chlorophyll content .The enzymatic activity of polyphenol oxidase and peroxidase enzymes was also measured according to Shi etal (2002)and Kim *etal*. (1988) . Disease incidence was calculated according to following equation.

$$Disease\ incidence\ (D.I) = \frac{Number\ of\ infected\ plants}{Total\ of\ plants}\ X\ 100$$

The disease severity was calculated according to the Mickenny equation (1923) Depending on scale consisting of four degrees0=leaves are intact;1 = slight yellowing ;2 = yellowing and wilting;3 = leaves are completely dead.Fig (1)

$$Disease\ Severity = \frac{Sum(No.of\ leaves\ in\ each\ degree\ X\ degree)}{No.of\ leaves\ Calculated\ X\ maximum\ scale\ degree}\ X\ 100$$

Fig(1) :-The description key.

Results and discussion:-

1-isolation of the fungus *F. oxysporum f.sp lycopersici* .

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The fungus*F. oxysporum f.sp lycopersici* was isolated from tomato plants (brought from the greenhouses of the College of Agriculture / Basra) showing signs of yellowing and wilting on the shoots with brown coloration that appears at the incision of the base of the stem and roots longitudinally. The fungus colony on PDA appear as white turned to a cream or pale yellow color. When the fungus was examined microscopically, microconidia and Macroconidia were seen with the presence of Chlamydospores. The microscopic characteristics of the fungus were identical to Leslie and Summerell (2006).

Also, the pathogenicity test of the fungus showed that *F.oxysporum f.splycopersici* isolate possesses a high pathogenic ability, as the percentage of tomato seed germination reached 26.67% compared with 78% in the control treatment. Fig (2).

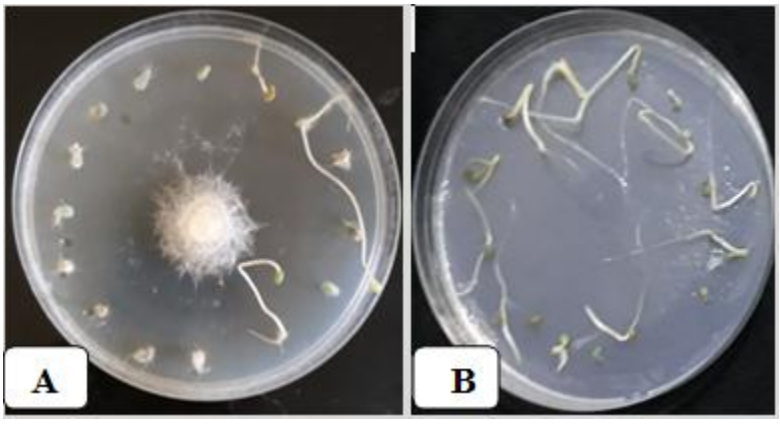

Fig (2) The effect of *F.oxysporum f.sp lycopersici* isolate on tomato seed germination.

2- Isolation of endophyte bacteria from the internal tissues of tomato plants and some associated weeds.

Eighty-two endophyte bacteria were obtained from different parts of the tissues of intact tomato plants and some weeds such as *Melilotus officinalis*, *Malva parvifllora*, *Amaranthus blitoides*, *Chenopodium album*and*Chenopodium album* respectively. And from different areas of Basra Governorate.

34 isolates obtained from Safwan area, 15 isolates from Al-Zubair area, 16 isolates from Al-Deir and 16 isolates from the green hoses of the college of

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Received 15 April 2021; Accepted 05 May 2021.

Agriculture/university of Basrah .Isolates of endophyte bacteria were purified to complete the subsequent experiments .

These results are consistent with several previous studies refer to isolating endophyte bacteria from the internal tissues of tomato roots and stems, including the study of Aydi-Ben Abdulla *et al.* (2016) in which he obtained 8 isolates from the internal tissues of tomato plants, belonging to the genus *Pseudomonas spp*, some of which showed an antagonistic ability against the fungus*F. oxysporuim f.sp lycopersici*. Kumar *et al*. (2013) used*Pseudomonas aeruginosa* BP35 isolated from the inner tissues of pepper plants in the biological control of the pathogen *Phytophthora capsici*.Yang *et al*. (2011) obtained 72 isolates of endophyte bacteria from tomato stem some of which such as *Brevibacillus brevis W4* possess high efficacy against the fungus *Botrytiscinerea*. Several other studies indicated that endophytic bacteria isolated from some weeds such as *Distichlis spicate* and *Pluchea absinthioides* possess antagonistic activity against phytopathogenic fungi (Zhang *et al*. 2019; Islam*et al* 2018) .

3- preliminary test of endophyte bacterial isolates against *Fusarium oxysporum f.splycopersici* (well and dual culture technique).

The results of the preliminary antagonistic test carried out by welltechnique showed that some endophyte bacterial isolated from the internal tissues of tomato plants and their associated weeds possessed antagonistic activity against *F.oxysporum f.sp lycopersici* (Fig3), these isolates include D-TR15 isolates isolated from tomato roots (Al-Deir) in which the zone of inhibition of the pathogenic fungus was 13 mm, while the zone of inhibition reached 12mm in D-CHST-79,A-ADST67, A-MeR38, and A-ch82,isolates, which were isolated from *C. album* stem,*A.blitoides* stem, *M.indicus* root and*C.album* leaves respectively, compared to 11 mm in the S-TR4, S-TRT79,Z-TCR13 and A-AmR68. Whiledual culture technique revealed that the isolate D-CHST 79 ,S-TRT79 and A-MeR38 which were isolated from *C.album*, , Tomato root and *Melilotus indicus* gave the best result in inhibition the growth of *F.oxysporum f.sp lycopersici* as the percentage of inhibition reached 45,45 and 40% respectively .The percentage of growth inhibition for other isolates ranged between 30--38%.The other isolates did not show an ability to inhibit the growth of the pathogenic fungus *F.oxysporum f.sp lycopersici*.Based on the results of this test, the above isolates were selected to

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complete the subsequent experiments.Aydi-BenAbdallah *et al*. (2016) indicated that endophyte bacteria isolated from the roots of healthy tomato plants inhibited the growth of the fungus *F. oxysporum f.splycopersici*. Islam *etal* (2018) showed that 6 isolateout of 35 endophytic bacteria included *Pseudomonas aeruginosa*isolated from the roots of different plants possessed high efficiency in controlling Fusarium wilt disease on the cucumber caused by *Fusarium oxysporum f. sp. Cucumerinum*.

The results of isolation endophyte bacteria from roots and other parts of tomato plant were in agreement with a study conducted by Elanchezhiyan *et al* (2018), who indicated that 23 isolates belonging to the genus *Bacillus sp*, *Azotobacter chroococcum* and *Serratiamarcescens* were isolated from the internal tissues of different plants without causing any damage to the plant ,and the bacterium *Bacillus sp* inhibited the growth of *F.oxysporuim f.sp lycopersici.*The antifungal activity of endophyte bacteria may be due to its ability to produce many secondary metabolite such as lipopeptide proteinPyocyanin and hydroxyphenazine (Kerr. *et al*.2014).Brader *et al*. (2014) also indicated that many species of endophytic bacteria produce alkaloid and other compounds such as Sespenine and anti-fungal spoxazomicins.

Table (1) The effect of bacterial isolates on inhibiting the growth of the fungus*F.oxysporum f.sp lycopersici.*

Source of isolates	%of fungal growth inhibition(dual	Zone of inhibition mm(Agar well)	Isolate symbol
Tomato roots(Safwan)	33.3	11	S-TR4
Tomato roots/Zubair	38	10	Z-TR14
C.album stem/Al-daer	45	12	D-CHST79
Tomato root/Safwan	45	11	S-TRT79
Tomato crwon /Zubair	33.3	11	Z-TCR13
A.blitoides stem/Agri college	33	12	A-ADST67

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 3119 – 3132
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Tomato root/Al-Deir	33	13	D-TR15
A.blitoides root/Agri college	28	11	A-AmR68
M.indicus/Agri college	30	12	A-MeR38
C.album leaves/Agri college	30	12	A-ch82
	2.8	1.81	L.S.D P=0.01

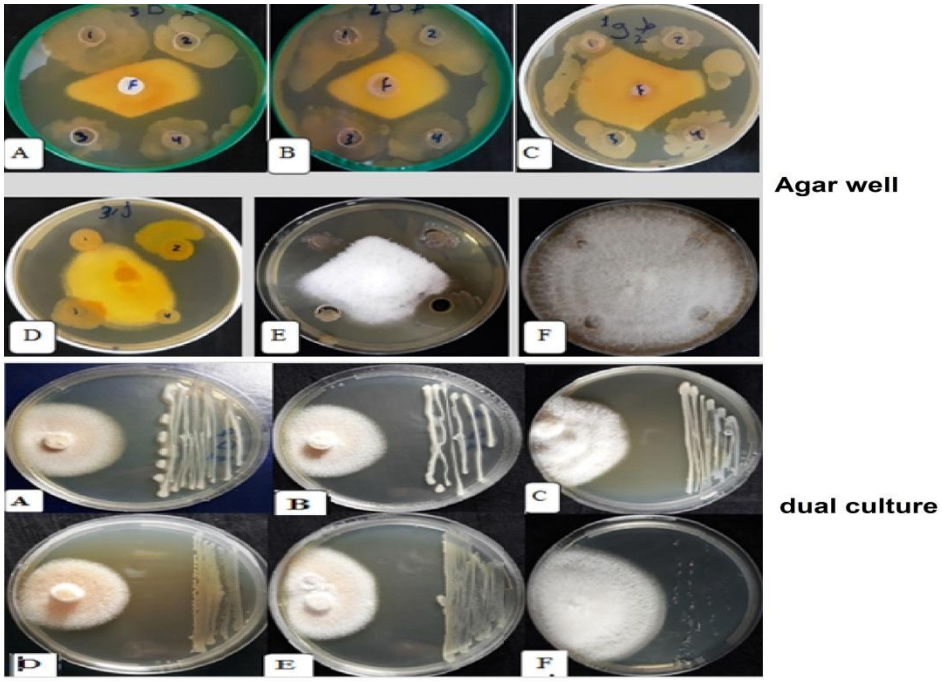

Fig (3) Effect of some endophytic bacteria in the growth of F.oxysporum f.sp lycopersici.A=D-CHST79 ;B=S-TRT79 ;C=Z-TCR13 ;D=A-ADST67;E=A-MER38 ;F=Control.

4-identification of endophytic bacteria isolated from internal tissues using the VITEk device.

The results of identificationof endophyitc bacteria by VITEK 2 deviceshowed that three isolates belong to *Psudomonasaeruginosa*, one isolate belong to *P.putida* while the other isolates belong to*Staphylococcus lentus*,*Enterobacter cloacaespp dissolvens*,*Alloiococcus otitis* and *Serratia marcescens* symbol and the source of isolates were listed in Table(2).Several previous studies indicated that the VITEk

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 3119 – 3132
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device is an appropriate tool in identification of fungi, yeasts and bacteria (Eigner *et al* . 2005,Funke, *et al* 1998).

Since the bacteria *Enterobacter cloacae*, *Alloiococcuus otiti*, *Staphylococcuslentus* and *Serratia marcescens* are known to cause some human health problems they were excluded from the subsequent experiments.

Table (2) Identification of Endophyte bacterial isolates isolated from some plants by VITEK 2 device.

Source of isolate	% of compatibility	The scientific name	Isolate symbol
Tomato roots(safwan)	% 88	*Staphylococcus lentus*	S-TR4
Tomato roots/Zubair	% 99	*Staphylococcus lentus*	Z-TR14
C.album stem/Al-Dier	% 99	*ssp dissolvensEnterobacter cloacae*	D-CHST79
Tomato root/safwan	% 99	*Psudomonas aeruginosa*	S-STR79
Tomato crwon /zubair	% 99	*Psudomonas aeruginosa*	Z-TCR 13
A.blitoides stem/Agri college	% 99	*Psudomonas aeruginosa*	A-ADST67
Tomato root/Deir	% 95	*Alloiococcus otitis*	D-TR15
A.blitoides root/Agri college	% 99	*ssp dissolvensEnterobacter cloacae*	A-AmR68
M.indicus/Agri college	% 99	*Psudomona putida*	A-MeR38
C.album leaves/Agri college	% 99	*Serratia marcescens*	A-ch82

5- The effect of some endophyte bacterial isolates on infection of tomato plants with the fungus*F.oxysporum f.sp lycopersici.*

The results of this experiment, Table (3), showed that the pathogen *Fusariumoxysporum f.sp lycopersici* caused an infection to all plants cultivated in

Annals of R.S.C.B., ISSN:1583-6258, Vol. 25, Issue 5, 2021, Pages. 3119 – 3132
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pots, as the disease incidence reached 100%, but the use of *Pseudomonasaeruginosa* and *Pseudomonas putida* by dipping the seedling roots in bacterial suspension reduced the negative effect of the pathogen, as the disease incidence was 41.7, 50, 41.7%, and 41.7% in *P. aeruginosa*-4, *P. aeruginosa-5*, *P. aeruginosa* 6 and *P.putida*-12, respectively, and disease severity was 22.9, 26.7, 24.9 and 25%, respectively, compared to 45.8%, in control treatment.It is also showed that the treatment of tomato seedlings with endophyte bacterial isolates led to an increase in the total chlorophyll rate in the leaves, which reached 6.39 in the treatment of *P.putida*, compared with 1.39 in the control treatment. On the other hand, the treatment of tomato seedlings with endophyte bacterial species increased Polyphenol oxidase and peroxidase activity in tomato tissues compared with the control treatment . which indicates the ability of the endophyte bacteria used to induce systemic resistance in plants(Rai *et al*., 2011; Ramamoorthy *et al*., 2002).Aydi -Ben Abdallah*et al*. (2016) showed that the bacteria *Pseudomonas spp*. isolated from the internal tissues of tomato plants to be highly effective in reducing infection with *F.oxysporum f.sp. lycopersici* and the bacteria produced salicylic acid and siderophores.Several studies have shown that the endophyte bacteria that live inside plant tissues can coexist and grow and work to induce systemic resistance or secrete some substances that inhibit fungi and enhance plant growth (Aydi- Ben Abdallah *et al*, 2020).

Table (3) The effect of endophyte bacterial isolates on the percentage and severity of Fusarium wilt disease (pot experiment).

Po activity unit/g wet weight	Ppo activity .unit/g wet weight	Chlorophyll mg/100 gm plant tissue	%Disease severity	%Disease incidence	Treatments
1.71	2.38	5.78	22.9	41.7	*P.aeruginosa 79 + F*
1.53	2.33	5.83	26.7	50.0	*P.aeruginosa 13*+F
1.77	2.14	4.51	24.9	41.7	*P.aeruginosa 67 + F*
1.96	2.43	6.39	25	41.7	*P.putida*- 38 + *F*
0.32	0.78	1.39	45.8	100	Control 1(F only)
1.25	1.75	4.35	00	00	Control 2(free from pathogen)
0.37	0.34	1.44		21.04	L.S.D .p=0.05

Ppo=polyphenol oxidase ; po=peroxidase

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