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Secondary invader bacteria associated with the red pest weevil infestation in date palm trees

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Abstract

The worldwide loss of date palm (*Phoenix dactylifera* L.) productivity is due to the infestation of the red pest weevil (RPW). The pest makes tunnels in the tree trunk and could be followed by secondary microbial infections causing oozing of fluid with a distinct fermentation odor. This study aimed at isolation and identification of secondary invader bacteria associated with the RPW and confirmation of their potential destructive effect on the tree. Four bacterial isolates were recovered from the infested date palm tissues, and they were identified based on phenotypic characteristics using the VITEK2 system. Three out of the four isolates were identified as *Pseudomonas aeruginosa*, and one isolate was identified as *Burkholderia cepacia*. All isolates caused rot of date palm germinated seeds (up to 40%), and wilt and root rot of date palm seedlings (40–100%). *Pseudomonas aeruginosa* (isolate No. 3) was the most aggressive isolate that involved 40% mortality and caused 100% of root rot incidence in date palm seedlings. *Burkholderia cepacia* showed the lowest degree of rot on the germinated seeds, percentage mortality, percentage root rot incidence of date palm seedlings. *Pseudomonas aeruginosa* (isolate No. 1) and *B. cepacia* caused a high loss in the weight of date palm petioles tissues (about 10%). The study threw light on the detrimental effects of secondary invader microbes associated with the RPW infestation. The study recommended an application of an integrated management program containing both antibacterial and insecticidal preparation in the management of the RPW to avoid a further negative effect on the palm trees.

1 | INTRODUCTION

Date palm (*Phoenix dactylifera* L., Family Arecaceae) is the most important tree cultivated in arid regions under water and salt stress in Africa, Asia, Europe, and the Americas (Chao & Krueger, 2007). A hundred million date palm trees cultivated in a million hectares of land worldwide are producing about 6.8 Tg of fruit (Al-Hamdany et al., 2011; FAO, 2007). Based on a FAO (2012) report, the highest producer countries of date palm fruits are Egypt (1,352,950 t), Saudi Arabia (1,078, 300 t), and the Islamic Republic of Iran (1,023,130 t).

Rhynchophorus ferrugineus, a red pest weevil (RPW), attacks date palm trees, coconut (*Cocos nucifera* L.), and ornamentals in Asia, Africa, Europe, and America (Howard et al., 2001; Yuezhong et al., 2009). In Egypt during 1992–1999, severe loss in date fruit production was caused by the infestation with RPW of 216,000 date palms (Murphy & Briscoe, 1999). Few genera of bacteria attack date palm plant causing deterioration such as sudden death by *Erwinia chrysanthemi* (Abdalla, 2001; Benjama, 1994), root rot by *Alcaligenes faecalis* and *Pseudomonas aeruginosa* (Ziedan et al., 2020). *Burkholderia cepacia* was first described in 1950 as the causal agent of sour skin disease on onion (*Allium cepa* L.) (Burkholder, 1950), banana finger-tip rot in Taiwan

Abbreviations: NA, nutrient agar; RPW, red pest weevil.

(Lee et al., 2003), bacterial fruit rot disease of apricot (Prunus armeniaca L.) in China (Fang et al., 2009), leaf streak of bird of paradise (*Strelitzia reginae* Ait.) in China (You et al., 2014) and a blight on millet [*Pennisetum glaucum*) (L.) R.Br.] with symptoms of yellowing, chlorosis, and a blight on leaves in Brazil (Conceição et al., 2019).

This study assumed that many microorganisms could be associated with this pest (RPW) and will be transmitted to the tree tissues during infestation and cause destructive diseases such as root rot, which could be involved in the death of the trees as an ultimate event of the disease cycle. The study focused on the roles of bacteria that follow the infestation of the RPW and determine their potential pathogenicity on the date palm germinated seeds and seedlings.

Core Ideas

- The study confirms the destructive effect of secondary invader bacteria in palms.
- *Burkholderia cepacia* and *Pseudomonas aeruginosa* were secondary invaders associated with red pest weevil.
- *P. aeruginosa* was an aggressive isolate involved in 40% mortality of palm seedlings.

2 | MATERIALS AND METHODS

2.1 | Isolation of bacteria from date palm trunk infested with red pest weevil

Ten grams of tissues from different parts of the date palm trunk (cultivar Zaghlol), infested with RPW (degraded and fermented tissues) from the Kafr El-Abida village, El- Mahalla Al- Kobra District, El- Gharbea Governorate, Egypt, were added to 90 ml of sterilized distilled water in 250-ml Erlenmeyer flask. After 30-min shaking, a serial dilution (up to 10^{-4}) of each sample suspension was prepared. One milliliter of each dilution was poured into a petri dish (9-cm in diameter), then 20 ml of Cetrimide agar medium (gelatin peptone 20.0 g L^{-1}, magnesium chloride 1.4 g L^{-1}, potassium sulfate 10.0 g L^{-1}, cetrimide 0.3 g L^{-1}, and 20 g L^{-1} of agar at pH 7.2) was poured as a selective medium for counting fluorescent *Pseudomonas* bacteria (Baron et al., 1994), and nutrient agar (NA) medium containing; (5 g of peptone, 3 g of beef extract/yeast extract, 5 g NaCl, 1% glycerol, and 15 g of agar in 1 L of distilled water, was used for counting the other species (Downes & Ito, 2001). The plates were incubated at 28 ± 2 ˚C for 2–3 d and the emerged colonies were counted. Single colonies were streaked on NA medium and incubated at 28 ± 2 ˚C for 2–3 d, and the pure cultures from single colonies were then kept at 4 ˚C for further studies.

2.2 | Identification of the bacterial isolates by VITEK2 system

Colonies of the bacterial isolates were streaked on NA medium supplemented with 1% glycerol, then they were incubated for 48–72 h at 28 ± 2 ˚C. The isolates were tested for the Gram stain reaction. Asingle colony was placed in a test tube containing 18 ml of 0. 85% sterile NaCl and adjusted to an appropriate density by measuring the optical density at 500 nm using a spectrophotometer. Fifteen microliters of each isolate were dispensed into each well of the microplates, then incubated at 28 ± 2 ˚C before identification according to cultural, physiological, and biochemical characterizations using VITEK2 systems (VITEK2 systems version 07.01). The interpretation standard was used according to the interpretation guideline of the Reference Laboratory for Drinking Water in Shobera El- Kima, Cairo, Egypt. The test kit card with the transferred suspensions was placed in the VITEK incubator. The VITEK system analyzes the card as the growth of the organism that occurs and gives an identity of the organism (Pincus, 2006).

2.3 | Potential pathogenicity of the bacterial isolates on date palm germinated seed

The inoculum of each bacterial isolate was prepared in concentration at 10^7 cell ml^{-1} from a fresh colony grown on nutrient broth medium and incubated for 2 d at 28 ± 2 ˚C. The isolates were tested on date palm germinated seeds between wet two layers of sterilized filter paper (Whatman No.1), which were incubated for 1 wk at 28 ± 2 ˚C. Five germinated seeds were inoculated with 10 ml of each bacterial isolate suspension (10^7 cell ml^{-1}) and were incubated in a petri dish (9-cm diam.). Five plates were used as replicates for each isolate. In the control treatment, the seeds were received 10 ml of sterilized distilled water and were incubated under the same conditions. The rotting percentage of the germinated seeds of date palm was recorded 15 d after treatments (Ziedan et al., 2015).

2.4 | Potential pathogenicity of the bacterial isolates on date palm seedlings

Six-month-old date palm seedlings were transplanted into 10 g sterilized peat moss in 250 conical flasks and inoculated with 10 ml of bacterial suspension (10^7cell ml^{-1}) of each

isolate as mentioned before. Four plants were cultivated in each flask, and three flasks were used per bacterial isolate. Three flasks free of bacterial infestation were used as a control. The flasks were incubated at 28 ± 2 °C and 85% of relative humidity for 30 d. All experiments were laid out in a complete randomized design and they were repeated twice (Ziedan et al., 2020).

2.5 | Estimation of root rot disease incidence and disease severity on root and shoot systems

Percentage of date palm seedlings mortality, disease incidence of root rot were determined after 30 d according to Ziedan et al. (2020). Disease severity on the shoot system was determined using a linear scale from 0 to 4; where 0 = no wilted leaf, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, and 4 = whole plant wilted. The disease severity on root system was estimated as follows: 0 = no discoloration on root, 1 = 1–25% brown discoloration of root 2 = 26- 50% brown discoloration, 3 = 51–75% brown discoloration, and 4 = 76–100% brown discoloration using the following formula:

$$\text{Disease severity} = \Sigma\,(n \times \mathrm{r})/N \times 100$$

where n = number of plants in each numerical disease grade, r = number of the disease grade, and N = total number of plants multiplied by the maximum numerical disease grade (Downes & Ito, 2001).

2.6 | Degradation potential of date palm trunk tissue caused by the bacterial isolates

The degradation potential of bacterial isolates was tested on pieces of date palm petioles. Fifty grams of date palm petiole' s tissues were sterilized by dipping in sodium hypochlorite 2% for 5 min, then they were washed with sterilized water three times to remove residue of the disinfecting agent and dried between two layers of filter papers. Five pieces were put in a sterilized plastic cage and were inoculated with 1 ml of cell suspension (1×10^7) of each isolate tested. In the control treatment, five pieces were treated with sterilized distilled water. All treatments were incubated at 28 ± 2 °C for 30 d, then the loss in petiole weight was determined according to Al-Hamdany et al. (2011).

2.7 | Statistical analysis

The data were initially examined for their normal distribution of errors using Shapiro–Wilk's W test and for homogeneity of variances using Levene's test. Data were analyzed for the significance of variation using one-way ANOVA. The means were compared using Duncan's multiple tests to identify the significant differences ($P < .05$) (Snedecor & Cochran, 1980).

TABLE 1 Bacterial count of date palm tissues infested by the red pest weevil (RPW)

Tissue sample	Bacterial count
	cell g^{-1}
Trunk tissues infested with RPW	3.0×10^4
Degraded trunk tissues	36.0×10^4
Fermented trunk tissues	2.0×10^4

3 | RESULTS

3.1 | Count and isolation of bacteria associated with date palm infected with the red pest weevel

Tissue samples of date palm trunk infested with the RPW that showed lysis and fermentation in trees' trunk tissues were used for counting the microbial communities (Table 1). The results proved the presence of bacteria in all examined samples of date palm tissues in considerable titer ($2.0–36.0 \times 10^4$ cell g^{-1}). The highest count of bacteria was associated with the lysed tissue; however, the lowest count was detected in the tissues infested with the RPW.

3.2 | Identification of the bacterial isolates

Pure cultures of the four bacterial isolates obtained from the degraded tissues of date palm trunk infested with RPW grown on NA medium supplemented with 1% glycerol. Based on the physiological and biochemical characteristics of the bacterial isolates using VITEK2 systems, as shown in Table 2, their identity was confirmed as *Pseudomonas aeruginosa* (three isolates) and *Burkholderia cepacia* (one isolate).

3.3 | Potential pathogenicity of bacterial isolates on germinated seeds of date palm

Data in Table 3 indicated that the four bacterial isolates caused rot of the germinated seeds of the date palm compared with the uninfected seeds (control). *Pseudomonas aeruginosa* isolates No. 1 and No. 2 were more aggressive than isolate No. 3 of the same species on the germinated seeds of the date palm (Figure 1). The disease incidence of root

TABLE 2 Phenotypic, biochemical characterization, and Gram stain reaction of four bacterial isolates using the VITEK2 system[a]

Features	Isolate No. 1	Isolate No. 2	Isolate No. 3	Isolate No. 4
Hydrogen cyanide (HCN) production	–	–	–	–
Ala-Phe-Pro-Arylamidase (APPA)	–	–	–	–
Adonitol (ADO)	–	–	–	+
L-pyrrolydonyl-arylamidase (PyrA)	–	–	–	–
L-arabitol (IARL)	–	–	–	+
D-cellobiose (dCEL)	–	–	–	+
Beta-galactosidase (BGAL)	–	–	–	–
Hydrogen sulfide production (H_2S)	–	–	–	–
Beta-N-acetyl-glucosaminidase (BNAG)	–	–	–	+
Glutamyl arylamidase (AGLTp)	+	+	+	–
D-glucose (dGLU)	+	+	+	+
Gamma-glutamyl-transferase (GGT)	+	+	+	–
Fermentation/glucose (OFF)	–	–	–	–
Beta-glucosidase (BGLU)	–	–	–	–
D-maltose (dMAL)	–	–	–	–
D-mannitol (dMAN)	+	+	+	+
D-mannose (dMNE)	+	+	+	+
Beta-xylosidase (BXYL)	–	–	–	–
Beta-alanin arylamidase pNA (BAlap)	+	+	+	–
L-proline arylamidase (ProA)	+	+	+	+
Lipase (LIP)	+	+	+	–
Palatinose (PLE)	–	–	–	(-)
Tyrosine arylamidase (TyrA)	–	+	+	–
Urease (URE)	–	–	–	–
D-sorbitol (dSOR)	–	–	–	+
Saccharose/sucrose (SAC)	–	–	–	–
D-tagatose (dTAG)	–	–	–	+
D-trehalose (dTRE)	–	–	–	+
Sodium citrate (CIT)	+	+	+	+

(Continues)

TABLE 2 (Continued)

Features	Isolate No. 1	Isolate No. 2	Isolate No. 3	Isolate No. 4
Malonate (MNT)	+	+	+	–
5-keto-D-gluconate (5KG)	–	–	–	–
L-lactate alkalinization (ILATk)	+	+	+	+
Alpha-glucosidase (AGLU)	–	–	–	–
Succinate alkalinization (SUCT)	+	+	+	+
Beta-N-acetyl-galactosidase (NAGA)	–	–	–	–
Alpha-galactosidase (AGAL)	–	–	–	–
Phosphatase (PHOS)	–	–	–	–
Glycine arylamidase (GlyA)	–	–	–	–
Ornithine decarboxylase (ODC)	–	–	–	–
Lysine decarboxylase (LDC)	–	–	–	–
L-histidine assimilation (IHISa)	–	–	–	–
Coumarate (CMT)	+	+	+	–
Beta-glucuronidase (BGUR)	–	–	–	–
O/129 resistance (comp. vibrio.) (O129R)	+	+	+	–
Glu-Gly-Arg-Arylamidase (GGAA)	–	(-)	(-)	–
L-malate assimilation (IMLTa)	+	+	+	–
ELLMAN (ELLM)	–	–	–	–
L-lactate assimilation (ILATa)	+	+	+	–
Gram stain reaction	–	–	–	–
Identity	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	*Burkholderia cepacia*
Similarity, %	99%	99%	99%	96%

[a] '+' corresponds to a positive response, '–' corresponds to a negative response, '(+)' indicates the reaction is mostly positive although occasional negative reaction may occur, '(–)' indicates reaction is mostly negative although occasional positive reaction may occur.

rot disease was recorded as 40% in the case of isolate No. 2 and No. 3, whereas the disease incidence was recorded as 25% in the case of isolate No. 1. *Burkholderia cepacia* recorded the lowest rot percentage on date palm germinated seeds (15%).

3.4 | Potential pathogenicity of the bacterial isolates on seedlings of date palm

Results in Table 4 demonstrated the ability of the tested isolates of both *P. aeruginosa* and *B. cepacia*, isolated from date

TABLE 3 The pathological potential of the bacterial isolates on the date palm germinated seeds

Bacterial isolate	Root rot incidence
	%
Pseudomonas aeruginosa (isolate No. 1)	25.0 b[a]
Pseudomonas aeruginosa (isolate No. 2)	40.0 a
Pseudomonas aeruginosa (isolate No. 3)	40.0 a
Burkholderia cepacia (isolate No. 4)	15.0 c
Control	00.0 d

[a]Values followed by the same letter are not significantly different at $P < .05$ according to Duncan's multiple range test.

FIGURE 1 Healthy date palm germinated seed (No. 0, leftmost) and rotten germinated seeds due to bacterial isolates *Pseudomonas aeruginosa* (No. 1, 2, and 3) and *Burkholderia cepacia* (No. 4) showing brown discoloration and maceration of root tissues

palm trunk tissues infested with the RPW, to cause a significant mortality percentage of 6-mo-old date palm seedlings after 30 d of inoculation compared with the control. Both isolates No. 2 and No. 3 of *P. aeruginosa* killed 40% of the seedlings, however, isolate No. 1 and *B. cepacia* were involved in killing 20% of the seedlings. The disease incidence percentage was recorded as 100% due to the infestation with the isolate No. 3 of *P. aeruginosa*. Furthermore, this isolate recorded the highest disease severity on the shoot and root systems as 2.4 and 3.5, respectively. On the other hand, *B. cepacia* and *P. aeruginosa* isolate No. 1 showed the lowest degree of disease severity on shoot and root of date palm seedlings (Figure 2).

3.5 | Degradation potential of the date palm tissues by the bacterial isolates

Data in Table 5 indicated that all bacterial isolates were able to degrade the petiole tissues of the date palm after 30 d from inoculation. *Pseudomonas aeruginosa* isolate No. 1 and *B. cepacia* caused the highest loss in the petioles weight followed by *P. aeruginosa* isolate No. 2 and No. 3.

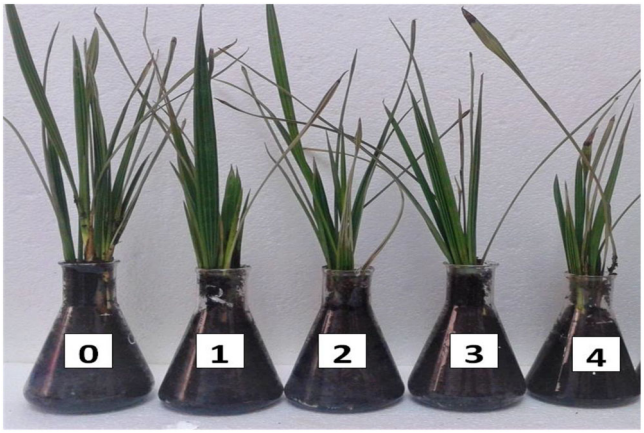

FIGURE 2 Healthy date palm seedling (No. 0, leftmost) and wilted seedlings due to bacterial isolates *Pseudomonas aeruginosa* (No. 1, 2, and 3) and *Burkholderia cepacia* (No. 4)

4 | DISCUSSION

The worldwide deterioration of date palm cultivation is mainly caused by the red pest weevil (*Rhynchophorus ferrugineus*) through making tunnels of softening the tissues inside trunks during their larval feeding by consuming a higher amount of the tissues (Mohamed et al., 2019), followed by secondary infection with saprophytic microorganisms, which produce distinct fermented odor in the infested trunk (Suma et al., 2014). Consequently, the synergistic destructive effect of both secondary invader microorganisms and RPW involves the death of the date palm trees for a short time (Howard et al., 2001; Kaakeh, 2006; Murphy & Briscoe, 1999).

In this study, four bacterial isolates associated with the date palm trunk infested with the RWP caused mortality, wilt of the shoot, and rotten of the root system of seedlings under artificial infection circumstances. Bacterial isolate No. 3 of *P. aeruginosa* caused the highest mortality percentage and root rot disease severity on the shoot and root systems of date palm seedlings. Meanwhile, *P. aeruginosa* isolate No. 1 recorded 100% root rot disease of the date palm seedlings. These results are in agreement with previous reports that approved infection of date palm plants with several pathogenic bacteria such as *Bacillus* spp. causing soft rot of date palm tissues (Leary et al., 1986), *Erwinia chrysanthemi* causing sudden decline (Abdalla, 2001; Benjama, 1994). Recently root rot disease of date palm plant caused by bacterial isolates of *Alcaligenes faecalis* and *Pseudomonas aeruginosa* in Egypt was approved (Ziedan et al., 2020). Interestingly, to the best of our knowledge, *B. cepacia* was reported as a root rot pathogen of date palm plant in this study for the first time. By reviewing the published data around the world, we did not find any information about the pathogenicity of this species on date palm, however, it was confirmed as a pathogen on other plant species such as sour skin disease on onion (Burkholder,

TABLE 4 Effect of bacterial isolates on mortality, root rot incidence, and disease severity rating of the date palm seedlings

Bacterial isolate	Mortality	Root rot incidence	Disease severity rating	
			Shoot system	Root system
	%			
Pseudomonas aeruginosa (isolate No. 1)	20.0 b[a]	80.0 b	1.8 c	1.8 d
Pseudomonas aeruginosa (isolate No. 2)	40.0 a	80.0 b	2.2 b	2.7 b
Pseudomonas aeruginosa (isolate No. 3)	40.0 a	100.0 a	2.4 a	3.5 a
Burkholderia cepacia (isolate No. 4)	20.0 b	40.0 c	1.8 c	2.0 c
Control	00.0 c	00.0 d	0.0 f	0.0 e

[a]Values in the same column and followed by the same letter are not significantly different at $P < .05$ according to Duncan's multiple range tests.

TABLE 5 Degradation potential of the bacterial isolates on the petiole of date palm after 30 d from infestation at 28°C

Bacterial isolate	Percentage reduction in weight of petiole tissues
	%
Pseudomonas aeruginosa (isolate No. 1)	10.25 a[a]
Pseudomonas aeruginosa (isolate No. 2)	8.32 b
Pseudomonas aeruginosa (isolate No. 3)	6.56 c
Burkholderia cepacian (isolate No. 4)	10.9 a
Control	00.0 d

[a]Values followed by the same letter are not significantly different at $P < .05$ according to Duncan's multiple range test.

1950), banana finger-tip rot (Lee et al., 2003), fruit rot of apricot (Fang et al., 2009), leaf streak of bird of paradise (You et al., 2014) and a blight on millet (Conceição et al., 2019).

Aside from the bacterial invaders, another cause of tissue degradation and fermentation of date palms is the toxic materials secreted or as a by-product of these invaders which move down to the roots and crown. Ethanol, acetic acid, acyl acetate, lactic acid, carbon dioxide, and hydrogen gas were among the main produced compounds and involved in fermentation and deterioration of the tissues (El-Sohaimy & Hafez, 2010; Monzer & Abd El-Rahman, 2003). Increasing concentration of ethanol greatly prohibited roots of *Vigna radiata* L. (Middleton et al., 1978) and acetic acid inhibited barley roots and shoot growth at seedling stage (Gussin & Lynch, 1982). In another study, yeast organisms like *Candida tropicalis* and *C. ethanolica* were isolated from the body of RPW and the infected tissues of date palm. These yeasts were responsible for the fermentation of some sugars including glucose, sucrose, and fructose (Abe et al., 2010).

During the last two decades, different control measures of integrated pest management (IPM) against RPW using insecticides, pheromone traps, and biocontrol agents were applied (El-Sufty et al., 2006; Faleiro, 2006; Salama et al., 2004), however, until now, no effective measure has emerged. We assume that the low effectiveness of any control measure could be due to the complexity of the disease, whereas the pest carries many secondary invader microorganisms involving in many other diseases including root rot and tissue deterioration of the internal tissues leading to extending the time of the infection and put many challenges towards using of effective control measures. From this point of view, our study threw light on the dangerous effect of the associated secondary invader microorganisms. This study suggests that to avoid further destruction in the tissues of the date palm, infested with the RPW, an appropriate bactericide should be applied as the main ingredient in the integrated disease management program of the RPW on date palm trees.

Our study identified both *B. cepacia* and *P. aeruginosa* as secondary invaders associated with the RPW that are causing root rot and wilt of date palm trees. *Pseudomonas aeruginosa* was reported as a more aggressive pathogenic bacteria than *B. cepacia*, however, both species caused a considerable degradation of the date palm tissues. The study pays attention to the dangerous effect of the secondary invader microbes accomplished with RPW that induces the deterioration of the tree's tissues. Therefore, new strategies should be considered for the management of RPW and we recommend using a mix of pesticides and bactericides to treat the infected trees.

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AUTHOR CONTRIBUTIONS

El-Sayed H. E. Ziedan: Conceptualization; Investigation; Methodology; Writing-original draft. Saad A. Alamri: Data curation; Writing-original draft. Mohamed Hashem: Conceptualization; Formal analysis; Writing-original draft; Writing-review & editing. Yasser S. Mostafa: Funding acquisition; Writing-review & editing. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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