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Full Length Research Paper

Study on the combined insecticidal effect of pyrethroid, *Azadirachta indica* and boric acid on the *Bacillus thuringiensis* efficacy in *Tribolium castaneum*

Kausar Malik*, Sidra Nazir, Amjad Farooq, Farkhanda Jabeen, Shagufta Andleeb and Mir Muhammad Ali Talpur

Department of the Zoology, Lahore College for Women University, Lahore, Pakistan.

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The red flour beetle (*Tribolium castaneum*) is a common pest insect known for attacking and infesting stored flour and grain. *Bacillus thuringiensis* (*B.t*) is a Gram positive, soil dwelling bacteria commonly used as a biological alternative to a pesticide. The present project was designed for the study of combined insecticidal effect of *B. t* along with boric acid, insecticide cypermethrin and the plant leaves powder, that is, *Azadirachta indica* against the *T. castaneum* to check the mortality rate of these insects and LC_{50} was calculated. Bacteria were isolated from rich soil, pulse dust, grain dust and its growth was checked on the T3 media by applying the microbial techniques. The results obtained show synergism when applied in combined concentration of *B.t*, boric acid, cypermethrin and powder of neem leaves in 1 g of diet. The combined concentration of each gave higher mortality to larvae and the percentage mortality was calculated as 33, 16 and 16% after 24 h, 41, 25 and 16% after 48 h and 50, 25 and 16% after 72 h in three different combined concentrations as compared to individual concentration. Likewise, in case of adults the percentage mortality was 33, 16 and 13% after 24 h, 41, 20 and 16% after 48 h and 41, 25 and 16% after 72 h of treatment.

Key words: *Tribolium castaneum, Bacillus thuringiensis*, boric acid, cypermethrin, efficacy.

INTRODUCTION

Tribolium castaneum is commonly known as red flour beetle attacked the store grain products like nut, beans, meal, flour, pasta, chocolate, spices, cereals, seeds, cake mix and also museum specimens (Via, 1999; Weston and Rattlingourd, 2000). Red flour beetles have mouthparts for chewing but they are not able for stinging and biting. These beetles may bring forth allergic responses but do not spread disease (Alanko et al., 2000). The origin of red flour beetle is of Indo-Australia and also found in temperate regions, but will endure the winter in sheltered places, where there is essential heat (Tripathi et al., 2001). The larvae and adults of *T. castaneum* prey on juvenile stages of rice moth, *Corcyra cephalonica.* Larvae and adults are big predators of eggs and pupae, so improving adult reproduction or larval development, thereby lessen competition for their descendants (Alabi et al., 2008).

In 1901, the bacterium *Bacillus thuringiensis* (*B.t*) was first discovered by Shigetane Ishiwata and after ten years it was rediscovered by Berliner. This *B.t* was isolated from infected larvae of *Anagasta kuehniella*, and this discovery led to the establishment of microbial insecticide or pesticide (Aizawa and Shigetane, 2001). *B.t* has 34 distinguished subspecies. There are two common groups of insecticidal crystal proteins, that is, Cry (crystal delta-endotoxins) and Cyt (cytolysins). Cry genes have been divided into four classes named CryI, CryII, CryIII and CryIV. Cyt genes have been classified into two classes (Hofte and Whiteley, 1989). Insecticidal activity of *B.t* mainly dependent on Cry proteins and it differ with the type of insect. *B.t* is also being used as biological pesticide against various insects belongs to the order like Homoptera, Lepidoptera, Coleoptera and Diptera (Cannon, 1993).

*Corresponding author. E-mail: kausarbasit7576@yahoo.com.

Chemical insecticides are helpful and effective method for controlling pests, as it is inexpensive to maintain the economic losses (Wadleigh et al., 1991). For the control of insects in food products, synthetic insecticides are used widely but on the other hand it was found that it affects the humans so it became necessary to lessen the use of insecticides in food products (Aldryhim, 1993). Pyrethrins are insecticides which are derived from the extract of flower called *Chrysanthemum cinerariaefolium*. Pyrethrum, the plant extract contains pyrethrin I and pyrethrin II collectively known as pyrethrins mainly used for the control of insects (Klaassen et al., 1996). Pyrethroids type I and II both slow down the nervous system of insects. This takes place in the nerve cell membrane through sodium ion channels. Most type II pyrethroids show its effect on the action of Gamma-aminobutyric acid (GABA) neurotransmitter (Costa, 1997).

Botanical pesticides are very eco- friendly and their use in crop field for the control of insects is very beneficial because it has minimum effect on the environment (Devlin and Zettel, 1999). Plant materials such as leaf extracts of neem (*Azadirachta indica*) and powder of neem seeds have been found to be very effective against different pests (Epidi et al., 2005). A neem seed extract, Azadirachtin has powerful insecticidal properties. It is naturally occurring anti- feeding insecticide (Sonata et al., 2005). Protection of storage grains and other food products from different pests has become a serious issue (Haq et al., 2005). It has been reported that due to insect infestations 9% of the world's grain production is affected (Tooba et al., 2005).

The strains of *B.t* isolated from marine environment are mostly dipteran, the reason is that larval stage of diptera lives in water (Iriarte et al., 2000). Three *B.t* strains that were isolated from stored tobacco residues and checked for their insecticidal activity against larvae of the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae). The bioassay was established and results showed that all strains caused approximately 60 to 80% mortality of the larvae after 7 days (Kaelin et al., 1999). Bio-pesticides can be categorized into three main groups: microorganism pesticides, biochemical pesticides and natural ingredient pesticides. *B.t* as bio-pesticide shows its characteristics as it has a narrow host range, eco-friendly and non-toxicity to plants and animals (Khetan, 2001).

Azadirachtin is mainly used only for its insecticidal properties. As insects are sensitive to smell, so they cannot bear the smell of neem oil. Azadirachtin disturbs the reproduction and growth in many pests. It is the most effective growth regulator. It resists or lessens the feeding activity of many nematodes as well as in insects (Ruskin, 1991). It was stated that powder of fresh neem seeds causes higher mortality as well as repellency to adults and third instar larvae of *Tribolium castaneum* as contrasted to stored neem seeds (Mohammed, 2005).

The use of pesticides has increased since the World War II. In UK, the active ingredient of pesticide gained its approval during the years between 1957 and 1995. Similarly the application of pesticides increased in North America. In the USA and in Canada, the increased usage of herbicides and insecticides occurred in the period of 1971-1987 (Sotherton and Holland, 2003).

Cypermethrin, a pyrethroid is chiefly used in UK for the control of ticks, lice and against the infestations caused by parasitic sea louse (*Lepeophtheirus salmonis*) and also used as scab on sheep. In the Salmon spawning rivers, cypermethrin has been found in the month of November and December (Moore and Waring, 2001).

MATERIALS AND METHODS

Rearing of insect

Each jam jars was filled 1/4th with sterilized mixture of wheat flour and some quantity of yeast extract as diet of *T. castaneum* and 40 adults were kept in each jar. The culture medium was placed in an oven at 60°C for 3 h, so that maximum reduction of contamination should be achieved. The larvae of *T. castaneum* were reared at 30±1 and 60±5% relative humidity (Saleem and Shakoori, 1984).

Collection of samples

There were three hundred samples of organically rich soil, grain dust, animal dung, pulse dust and bird droppings were collected from different areas of Lahore in sterile plastic bags or properly labelled glass jars.

Technique for the isolation of *B. t*

For the isolation of *B.t* all the 300 samples were processed according to technique of Martin and Travers (1989) as illustrated by Makhdoom (1997). For the processing of samples LB broth media and LB agar were used. One gram of each sample was weighed and suspended in 10 ml of LB medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 5 g/L) containing 0.2 M sodium acetate in sterilized test tubes and vortexed for 5 min in the laminar flow hood till a homogenized mixture was obtained. In order to collect the supernatant, the test tubes were kept for 30 min at room temperature and then filtered it. In sterile Eppendorfs tenfold serial dilution (10^{-1} to10^{-6}) were made from the supernatant as 100 µl supernatant and 900 µl of distilled water was added. To give heat shock for the isolation of *B.t* the dilutions were kept in water bath for 10 min at 80°C. 200 µl of samples was poured on LB agar plates; spread it with a sterile spreader and after that incubated them for 24 h at 32°C.

Screening of *B. t*

To study the colony morphology of the isolates, LB medium was used because the growth of *B.t* best occurs in LB medium. The growth on LB agar plates were checked after 24 h and only those colonies were taken which showed similarity to *B.t* like off white colour, entire margin, rich and dry growth of colony. These colonies were streaked again on LB agar plates and then incubated for 24 h at 32°C. After incubation, different morphology of the colonies was obtained as compared to the origin of their isolates. So from the

pure culture a single colony was picked for smear preparation and for the further identification of *B.t* spore staining and Gram staining were performed.

Staining and biochemical characterization of *B. thuringiensis*

Endospore staining and Gram staining as well as biochemical tests like starch hydrolysis were performed for the *B.t*. isolates (Karnataka, 2009).

Endospore staining

According to spore staining method of Schaeffer and Fulton (1933), approximately 72 h old culture of *B.t* was used along with malachite green and fuchsin. First of all, smear of cultures were prepared which contained crystals and spores and spread it on the slide and then air dried it after that heat fixed the smears. The slides were kept in water bath and poured the stain malachite green (5%) with the help of dropper for 15 min. Then the slides were flooded with the second stain, that is, fuchsin for 10 min. The slides were observed under the microscope at 100X with oil immersion.

Gram staining

Gram staining is the method that is used for the differentiation of Gram positive and Gram negative bacteria. This method was first introduced by Hans Christian Gram (1853-1938) (Gram, 1884). In this procedure 24 h old culture of *B.t* was taken on the slide and fixed it on the slide by heat. A thin smear was formed on the slides after that with the help of dropper crystal violet stain was poured on it for 1 min. Slides were washed with iodine, the stain was poured off and dried for 30 sec. The slides were washed with tap water till the decolouration occurred. Right after that the slides were flooded with the safranin stain for 1 min and again washed with tap water. Again the slides were observed under 100X power of microscope along with oil immersion.

Starch hydrolysis test

From LB agar plates, picked a colony of isolate by a sterile loop and streaked it on the potato starch plate. The plate was incubated for 4 days at 30℃ and then the plate was flooded with 1: 5 dilution of Iodine solution. The starch that was hydrolysed showed the formation of clear zone and the unchanged starch appeared blue black in colour.

Growth of B.t. in 7% Sodium Chloride

The use of 7% (w/v) trypticase salt was necessary in this test. Inoculated the test tubes which contained LB medium and selected strains. After incubation for 14 h at 37℃ the turbidit y was observed.

Preparation of media

B.t cells were grown on Petri plates containing T3 medium. The composition of T3 medium was 1.5 g of yeast extract, 3 g of tryptone, 2 g of tryptose, 0.005 g of $MnCl_2.2H_2O$, 15 g of agar/L and 2.5 ml of 1 M potassium phosphate (pH 6.9). All these chemicals were dissolved in distilled water.

Chemical bioassay

For each treatment, three replicates of each concentration were used. For the boric acid the three different concentrations were adjusted, that is, 0.05, 0.10 and 0.15 g along with per gram diet of *T. castaneum* in the glass vials and introduced 20 adults in it. After three days the mortality was checked. The mortality data was noticed in such a manner like every 24, 48 and 72 h, respectively.

Insecticidal plant bioassay

Preparation of leaf powder

Fresh and mature leaves of *A. indica* were selected for their insecticidal activity or as grain protactants or repellents against *T. castaneum.* These neem leaves were collected from the Lawrence garden, Lahore. These leaves were washed with distilled water and dried it. After that the dried leaves were grinded in grinder and the dry powder was kept in air tight glass jars.

The selected concentration of plant powder 1.5, 2.0 and 2.5 g were mixed as per gram diet of red flour beetle. Each concentration had three replicate and introduced 20 adults and larvae in each glass vial. The control sample was maintained without the neem powder. At regular interval of 24 h the mortality data was checked.

Insecticide bioassay

The test insecticide was cypermethrin commercially available of 10 EC. In the control only acetone was applied with diet. The different concentrations were 0.05, 0.15 and 0.25%. One ml of each concentration was dropped into the vials and spread it throughout the vial and after evaporation of acetone 20 adults were introduced in each dried vial along with per gram diet and covered it tightly.

***B.t.* bioassay**

The collected pellet of *B.t.* in falcon tube was mixed with distilled water and centrifuged at 300 rpm for 5 min. This centrifugation is repeated thrice in centrifugation tubes till all the media was washed away and the supernatant was discarded. In the end of the tube the pellet was obtained which was dried in an oven.

The dried pellet was taken and crushed it in pestle mortar to form the fine granule like powder. The concentration of *B.t*. is adjusted like 1.0, 1.5 and 2.0 g and mixed it with per gram diet, that is, 0.8 g semolina and 0.2 g of yeast extract. These concentrations were poured in glass vials and introduced 20 adults and third instar larvae in each vial separately. Mortality was observed after three days and by probit analysis SPSS programme the LC_{50} was counted.

Combined bioassay

Bioassay was carried out in combination with *B.t*, boric acid, cypermethrin and powder of neem leaves. Three different combined concentrations were prepared in separate glass vials like first concentration was 1.0 g *B.t,* 0.05 g boric acid, 0.05% cypermethrin and 1.5 g neem leaves powder as along with 1 g of diet. 20 adults and larvae were introduced in each vial. Total 60 larvae were used in each combined concentration and same way 60 adults were used for adult bioassay. Likewise second concentration was made 1.5 g *B.t,* 0.10 g boric acid, 0.15% cypermethrin and 2.0 g neem leaves powder in 1 g diet and third concentration was 2.0 g *B.t,* 0.15 g boric acid, 0.25% cypermethrin and 2.5 g neem leaves powder were mixed in 1 g of diet. For each combined concentration the bioassay was carried out for larvae and then for adults. This bioassay was also in triplicated form. The mortality data was checked after every 24 h. The combined percentage mortality was

calculated and compared it with individual mortality of larvae and adults.

RESULTS

Total 300 samples were collected from different sources like organically rich soil, grain dust, animal dung, pulse dust and bird droppings to isolate the *B.t.* All the *B.t*. samples were bioassayed and the most toxic strain (Sid1) was selected which gave highest mortality against the larvae as well as adults of *T. castaneum.* This Sid1 strain of *B.t.* was found more abundantly in grain dust and pulse dust.

After the selection and isolation of *B.t*. strains the further step was the identification of the bacteria. The growth on the LB agar plates gave the colony morphology of *B.t.* like off white colour, entire margin, rich and dry growth.

Bioassay of larvae

When the *B.t,* boric acid, cypermethrin and *A. indica* were combined in their different concentration the mortality of *T. castaneum* larvae was increased as compared to individual bioassay.

In this combined bioassay the different concentrations were mixed and checked it against the larvae for 24, 48 and 72 h of treatment. The results showed that the rate of mortality of larvae was decreased when the bioassay was carried out individually but when all the doses were combined it enhanced the mortality rate. The Percentage of dead larvae was increased as compared to the individual Percentage of *B.t,* boric acid, cypermethrin and *A. indica* at different concentrations (Table 1).

Bioassay for *T. castaneum* adult

In the combined bioassay the mortality of the adults was high as compared to individual bioassay of *B.t,* Boric acid, cypermethrin and *A. indica.* When the bioassay was carried out after 24, 48 and 72 h it was observed that mortality rate of adults was increased when the different concentrations of *B.t,* boric acid, cypermethrin and *A. indica* were mixed. The percentage of mortality was also high when the concentrations were mixed as compared to individual concentration of each (Table 2).

All the bioassays were carried out in triplicate form and every bioassay was repeated three times to check the accuracy of results.

DISCUSSION

Bacillus thuringiensis proved to be very effective bacteria against the *T. castaneum* larvae and adults as it gave high mortality for both. The Sid1 strain of *B.t* showed its effectiveness against the stored product pests like *T. castaneum,* in the same way it may be effective against other pests included in order Lepidoptera, Hymenoptera, Diptera, Homoptera and Dictyoptera. In the present study even low as well as high concentration of *B.t* gave mortality to larvae and adults; although the mortality rate of larvae was high because they were of small size and *B.t* proved to be more effective for larvae in their third instar stage. The LC_{50} value was also high in case of larvae as compared to that of adults.

In this study boric acid was used against the *T. castaneum* larvae and adults to check the mortality rate of both and also observed the LC_{50} value to estimate that in which concentration of the boric acid was effective against larvae and adults. As boric acid was harmless to humans, there was no detrimental effect while the experiment was conducted, but it proved to be very toxic against the *T. castaneum* larvae and adults. Even very low concentration, that is, 0.05 g of boric acid gave mortality of larvae and adults in 24 h of treatment and the value of LC_{50} was high, which is, 0.12 g/g for larvae and 0.16 g/g for adults. When cypermethrin was used in different concentrations for both larvae and adults the results after 24, 48 and 72 h were like this; the LC_{50} values were 0.49, 0.60 and 2.03% against *T. castaneum* adult and 0.42, 0.53 and 0.54% for larvae.

The powder of neem leaves was used against *T. castaneum* larvae and adults. It showed mortality against larvae and adults but not as much effective as compared to *B.t,* boric acid and cypermethrin. The mortality was not observed in 24, 48 and 72 h but in 3 weeks of treatment.

The one reason for low mortality was that it may be the repellent effect of neem leaves against the larvae and adults. The LC_{50} values were 4.40 and 6.17 and 7.05 g/g in 1, 2 and 3 weeks at three different concentrations 1.5, 2.0 and 2.5 g. The LC_{50} values in case of adults at same concentrations were 4.32, 4.60 and 5.35 g/g. The mortality rate of larvae was high as compared to the adults.

In the present study when different concentrations of *B.t,* boric acid, cypermethrin and *A. indica* was applied against the *T. castaneum* larvae and adults, the results showed that higher mortality was observed in combination of the concentrations as compared to the individual concentration. In case of larvae, the bioassay was carried out at combined concentrations like 1.0 g *B.t,* 0.05 g boric acid, 0.05% cypermethrin and 1.5 g neem leaves powder in 1 g diet for 24, 48 and 72 h of treatment. The combined percentage mortality was observed as 33 % in first 24 h as decreases in 48 and 72 h because with the passage of time mortality rate decreased as the effectiveness of different components was less. Likewise another concentration was set in combined form which was 1.5 g *B.t,* 0.10 g boric acid, 0.15% cypermethrin and 2.0 g neem leaves powder in 1 g diet, it also gave high combined mortality 41, 25 and

Table 1. Observed and percentage mortality of *Tribolium castaneum* larvae after 24, 48 and 72 h of treatment with different concentration of *Bacillus thuringiensis,* boric acid, cypermethrin and *Azadirachta indica* in combination.

Time interval (h)	Combine and individual concentration mixed with diet	Observed mortality (D/T)	Percentage mortality (%)
First concentration			
24	Control	0/60	0
	B.t 1.0 g	15/60	25
	Boric acid 0.05 g	12/60	20
	cypermethrin 0.05%	10/60	16
	Neem 1.5 g	9/60	15
	Combine	20/60	33
48	Control	0/60	0
	B.t	9/60	15
	Boric Acid	7/60	11
	cypermethrin	9/60	15
	Neem	6/60	10
	Combine	10/60	16
72	Control	0/60	0
	B.t	3/60	5
	Boric Acid	5/60	8
	cypermethrin	7/60	11
	Neem	3/60	5
	Combine	10/60	16
Second concentration			
24	Control	0/60	0
	B.t 1.5 g	17/60	28
	Boric acid 0.10 g	15/60	25
	cypermethrin 0.15%	11/60	18
	Neem 2.0 g	10/60	16
	Combine	25/60	41
48	Control	0/60	0
	B.t	8/60	13
	Boric Acid	8/60	13
	cypermethrin	10/60	16
	Neem	5/60	8
	Combine	15/60	25
72	Control	0/60	0
	B.t	4/60	6
	Boric Acid	6/60	10
	cypermethrin	9/60	15
	Neem	5/60	8
	Combine	10/60	16
Third concentration			
24	Control	0/60	0
	B.t 2.0 g	20/60	33
	Boric acid 0.15 g	18/60	30
	cypermethrin 0.25%	20/60	33
	Neem 2.5 g	15/60	25
	Combine	30/60	50
48	Control	0/60	0
	B.t	10/60	16
	Boric Acid	8/60	13
	cypermethrin	12/60	20
	Neem	8/60	13
	Combine	15/60	25
72	Control	0/60	0

Table 1. Contd.

	B.t	4/60	6
	Boric Acid	5/60	8
	cypermethrin	8/60	13
	Neem	4/60	6
	Combine	10/60	16

Table 2. Observed and percentage mortality of *Tribolium castaneum* adult after 24, 48 and 72 h of treatment with different concentration of *Bacillus thuringiensis,* boric acid, cypermethrin and *Azadirachta indica* in combination.

Time interval (h)	Combine and individual concentration mixed with diet	Observed mortality (D/T)	Percentage mortality (%)
First concentration			
24	Control	0/60	0
	B.t 1.0g	12/60	20
	Boric Acid 0.05 g	13/60	21
	cypermethrin 0.05%	12/60	20
	Neem 1.5 g	4/60	6
	Combine	20/60	33
48	Control	0/60	0
	B.t	6/60	10
	Boric acid	7/60	11
	cypermethrin	7/60	11
	Neem	4/60	6
	Combine	10/60	16
72	Control	0/60	0
	B.t	3/60	5
	Boric acid	5/60	8
	cypermethrin	6/60	10
	Neem	2/60	3
	Combine	8/60	13
Second concentration			
24	Control	0/60	0
	B.t 1.5 g	13/60	21
	Boric acid 0.10 g	13/60	21
	cypermethrin 0.15%	10/60	16
	Neem 2.0 g	8/60	13
	Combine	25/60	41
48	Control	0/60	0
	B.t	7/60	11
	Boric Acid	7/60	11
	cypermethrin	10/60	16
	Neem	6/60	10
	Combine	12/60	20
72	Control	0/60	0
	B.t	3/60	5
	Boric Acid	5/60	8

Table 2. Contd.

	cypermethrin	8/60	13
	Neem	3/60	5
	Combine	10/60	16
Third concentration			
24	Control	0/60	0
	B.t 2.0 g	18/60	30
	Boric acid 0.15 g	16/60	26
	cypermethrin 0.25%	20/60	33
	Neem 2.5 g	10/60	16
	Combine	25/60	41
48	Control	0/60	0
	B.t	6/60	10
	Boric Acid	9/60	15
	cypermethrin	10/60	16
	Neem	8/60	13
	Combine	15/60	25
72	Control	0/60	0
	B.t	3/60	5
	Boric Acid	4/60	6
	cypermethrin	5/60	8
	Neem	4/60	6
	Combine	10/60	16

16% in 24, 48 and 72 h of treatment as compared to the individual mortality. The third and last combined concentration was 2.0 g *B.t,* 0.15 g boric acid, 0.25% cypermethrin and 2.5 g neem leaves powder against *T. castaneum* larvae was applied. It also gave higher mortality in combined concentration as 50, 25 and 16% in 24, 48 and 72 h. It was also observed that with the increase in concentrations the mortality rate was also increased as at third combined concentration the percentage mortality was higher as compared to first and second concentrations.

The same combined concentrations were applied to *T. castaneum* adults in 24, 48 and 72 h. The percentage mortality was observed in combined concentration was high as compared to individual concentration but percentage mortality was not as high as in case of larvae in combined bioassay. At first combined concentration the percentage mortality of adults were 33, 16 and 13% and in second combined concentration 41, 20 and 16% but in the third and high concentration the percentage was increased as 41, 25 and 16% in case of adults.

In this study *B.t,* boric acid, cypermethrin and *A. indica* showed their insecticidal effect on the individually as well as combined against the *T. castaneum. B. thuringiensis* proved to be very effect bacteria against the stored product pests. Boric acid, cypermethrin and *A. indica* increased the efficacy of *B.t* against the *T. castaneum* larvae as well as adults. As the *B.t* and *A. indica* are biopesticides and are very beneficial for the control of variety of insect pests and also have benefits for humans. The crystal proteins of *B.t* had insecticidal properties against different pests. One crystal protein may be effective for many different kinds of pests. Likewise boric acid is a chemical and cypermethrin an insecticide which have no residual effects and safe for humans and other mammals as well as for the whole environment. In godowns the spray of insecticide kept the grain products safe from the different stored product pests. Different kinds of formulations were made from these biopesticides which helped in for the control of not only stored product pests but for the control of other forest, agriculture and household pests.

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