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Effect of Stem Gall Disease Caused by *Protomyces macrosporus* on Biochemical Changes of Coriander

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ABSTRACT

Aim: *Stem gall diseases have become major economic significance. The disease manifests itself in the form of galls on stem, branches, leaves, petioles and fruits, causes 15-20 per cent yield loss and deteriorates quality of seeds. Quantitative biochemical changes are required to determine for development of resistant variety against stem gall*

Methodology: *Stem gall symptoms were regularly observed during whole crop growth stage from appearance of symptom on different parts of the coriander plants. The leaves, stems, inflorescence, petioles and fruits showing characteristic symptoms were separately collected and kept in rough dry envelopes and marked clearly mentioning location, infected parts, variety, reaction types, date of collection etc. and brought to the laboratory for identification of the pathogen and used for the study of biochemical changes such as total phenol, tannins, flavonoids and Saponins*

Results: *The stem gall symptoms were observed as small tumour-like swellings on all herbaceous parts of the affected plants, namely, stems, petioles, flower stalks and leaves. The mycelium of Protomyces macrosporus was septate, hyaline, irregular, branched, 2 to 5 µm thick, 36.50 µm length and 35.00 µm width. Intercellular and multinucleate mycelium's are formed Chlamydospores, which are globose to ellipsoid, thick-walled, three-layered membrane and smooth with brownish colour. Mature Chlamydospores were multinucleate and measured 60-70 µm x 50-60 µm in diameter. Severely infected plant parts have synthesized low amount of phenol, tannin, flavonoid and saponin content, whereas comparatively high amount of these compounds are found in less infected plant parts or healthy one*

Interpretation: *Stem gall infected plant parts were immediately accumulate total phenol, tannin, saponin and flavonoids following the pathogenic attack in resistant varieties, whereas susceptible varieties did not accumulate significantly higher amount of these substances.*

Key words: *Stem gall, Coriander, Symptoms, Chlamydospore, Spread, Bio-chemical*

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INTRODUCTION

Coriander is grown in eastern part of Uttar Pradesh for both green leaves and seed yield. The crop is suffering number of biotic stresses *viz*; Stem gall, powdery mildew and wilt disease with causing yield loss upto 15-25%[15]. Among them, stem gall disease is causing 20.8percent yield losses in eastern part of Uttar Pradesh[11,25,27]. Recent past, incidence of stem gall disease has increased significantly and become a major limiting factor for successful cultivation of coriander[14,25]. The genus *Protomyces macrosporus* Unger are obligate parasites within the *Apiaceae* family causing galls on stems, leaves, flowers and fruits. On the leaves galls are restricted to petiole, midrib, veins and veinlet's[6]. In these galls ascogenous cells are present. Ascogenous cells are spherical to sub spherical with thick and smooth walls, formed intercalary in the intercellular mycelium throughout the infected tissue[9]. The success or failure of the pathogen to infect a new crop is partly determined by its survival and transmission from season to season. Recurrence of this disease was indicated earlier through the infected plant debris and over summering in the field that provide a source of primary inoculums in the following crop season[17]. The Chlamydospores (resting spores) released from the decayed host tissues after the monsoon rain and provide the primary inoculums. The possibility of survival of free Chlamydospores in the soil has not been indicated due to high temperature prevailing during the summer months (May to June) in this region[24]. Annual addition of fresh Chlamydospores and the blastospores into the soil through intensive cultivation of susceptible host crop are recurrence of the disease in the same field is led to explore the possibility of soil and infected plant debris for the transmission of pathogen. The bio trophic phase of mycelium depends on the host tissue for nutrients to sustain its growth and development. The marked characteristic of the parasitic life style of plant pathogen is the unidirectional transfer of nutrients from the host to pathogen[28]. Biochemical studies of plant pathogen interaction revealed that soluble sugar, starch, chlorophylls, phenol and tannin content were observed higher level in healthy leaves compared to pathogen infected leaves[2]. Only scanty information is available for biochemical changes in coriander plants during the growth of *Protomyces macrosporus*. The aim of this study was to increase knowledge regarding the physiological and biochemical changes during the occurrence of stem gall disease and try to define changes in phenols, flavonoids and tannins that might be related to the potential antioxidants and may be efficient as preventive agents in the pathogenesis of same disease.

MATERIALS AND METHODS

Diseased samples were collected from the coriander crop grown at Vegetable Farm, Narendra Deva University of Agriculture and Technology, Narendra Nagar (Kumarganj), Faizabad (U.P.) during 2014-2015. Stem gall symptoms were regularly observed during whole crop growth stage from appearance of symptom on different parts of the coriander plants. The leaves, stems, inflorescence, petioles and fruits showing characteristic symptoms were separately collected and kept in rough dry envelopes and marked clearly mentioning location, infected parts, variety, reaction types, date of collection etc. and brought to the laboratory for identification of the pathogen and used for further studies.. The samples were dried for 24 hours in shade in order to remove excess surface moisture. After drying the samples, were kept in B.O.D. incubator in paper envelope and temperature maintained 6-8 ^{0}C.Identification of the disease was done through visual symptoms appeared on infected plants and the pathogen of stem gall showing symptom was identified on the basis of available standard literature[6,22]. Morphological characteristics of the fungi were observed under macroscopic slide. Slide was prepared by cross and longitudinal sections of naturally infected leaves / stems and observed in drop of 50% lactic acid coloured with methylen blue. Observations were carried out using the Olympus light microscope fitted with micro photographic equipment's. The ascogenous cells were measured as described by Parmasto and Parmasto[22]. The morphology of *Protomyces macrosporus* isolates was characterized with

reference. Characters of mycelia and ascogenous cells viz. colour, septation, branching patterns size presence and absence in infected plants through microscopic examination. Resting spores/Chlamydospores characters i.e. shape, size and colour was also studied under microscope with the help of stage and ocular micrometer[4].

Spread of the Pathogen

Infected plant materials viz. infected plant debris, infected galled seed; infected plant seed without gall and contaminated soil (previously stem gall infected fields) were collected during March, 2015 at the regular stem gall disease occurring coriander fields. The plant materials were air dried and stored at room temperature from March to October (2015). All the infected material crashed with the help of pestle and mortar and mixed in sterilized soil before filling the pot. Soil of infected field and sterilized soil (control) were filled in separate pot. The susceptible coriander variety NDcor-2 was sown in each treatment with three replications. Pots were watered regularly. Observation of disease appearance, plant height, no. of branch's/plant, seed yield/plant, and no. of Chlamydospores/g soil and dry weight of straw were recorded after two month of sowing.

Count the Chlamydospores/g soil

One gram of soil was collected from each replication in the depth of 1 inch, 2 inch and surface at the distance of 2 inch, 3 inch and plant base. The soil were kept in envelops separately with marked of date, depth and distance and brought to laboratory for count of Chlamydospores. Count of Chlamydospores in per gram of soils was done under microscopic slides. Before the counting of Chlamydospores soil was mixed in 10 ml sterile distilled water, the 0.1 ml of uniformly dispensed suspension smeared over a marked area on a micro-scopic slide and examined the number of chlamydospore/g of soil.

Biochemical study

Infected and healthy plant parts *viz*. leaves, stem, seed and inflorescence were collected from stem gall infected coriander field to study the biochemical changes such as total phenol, tannins, flavonoids and saponins. The methodology are described as under-

Total phenol

Procedure

The healthy and infected plant parts viz. leaves, stem, inflorescence, and seed were directly selected from the field for the estimation of total phenol. One gram of dried sample was weighted, cut into small pieces and then placed in smearing methanol until the green colour was extracted. Leaves tissues were homogenized after decanting the methanol. These homogenized tissues were again boiled in methanol for further 5 minute and then filtered. Residual material was washed with 80% acidified (0.1% HCL conc.) methanol. Methanol was evaporated using a rotavapour and the aqueous layer was collected to adjust the final volume as ml/g of weight with distilled water. The aqueous portion of the extract was then washed with n-hexane to remove the green colour. Total phenols were estimated using Folin-Ciolcalteu reagent, according to the modified method. Place the tubes in boiling water for one minutes allowed to cool and measured the absorbance at 650 nm against a reagent blank. Standard curve was prepared using different known concentration of catechol. The absorbance was measured in a spectrophotometer at 650nm.

Calculation

From the standard curve find out the concentration of phenols in the test sample and express as µg phenols/g material. The total phenols are calculated by the following formula.

$$\mu g/g = \frac{\text{G.F. x O. D. x Total volume}}{\text{Aliquot taken x Weight of sample x 1000}}$$

G. F. = Graph factors

O.D. = observance density

Estimation of tannin content

Collection and preparation of plant solution

Healthy and infected stem gall of coriander plant parts were collected at the time of flowering and fruiting. The crude powders of the leaves, stem, inflorescence and seed were prepared for photometric determination of tannins. The standard procedure was followed by folin-Denis method. Powdered material of each (0.05g) was transferred into 250ml conical flask and adds 75ml water. Heat the flask gently and boil 30 minutes. The solution was centrifuged at 2000rpm for 20 minutes and collects the supernatant in 100ml volumetric flask and make up the volume. Then transfer 1.0ml of extract sample into 100 ml volumetric flask containing 75ml distilled water, and add 5.0ml of Folin-Denis reagent and 10ml sodium carbonate solution (sodium carbonate, 350g was dissolved in 1000ml water at 70 ^{0}C temperature), solution was allowed to stand overnight and then it was filtered through glass wool and dilute it with 100ml of distilled water and shaken well. Read the absorbance at 700nm after 30minutes against blank (water).

Preparation of standard curve

10ml of standard tannic acid solution (100g of tannic acid was dissolved in 100 ml distilled water) was made up with distilled water. 1-10ml aliquots were taken in clean test tubes of 0.5ml Folin-Denis reagent and 100ml of sodium carbonate solution was added to each tube. Each tube was made upto 10.0ml with distilled water. The reagents add in each tube and after about 30 minutes read the absorbance at 700nm against blank reagent.

Estimation of flavonoids

Total flavonoids were determined according to the methods of Nabavi *et al*[18]. Powdered sun dried leaves, stem, inflorescence and seed (1.0 g each) were extracted in a sox let extractor with 10 mL 80% methanol and shaking for 2 h. Total flavonoids extract (0.4 mL) was added to 4 mL of distilled water. Then 0.3 mL of 5% $NaNO_2$ was added. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. After 6 min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL with distilled water. The absorbance was measured at 510 nm against a blank reagent. Catechin used to prepare the standard curve. The flavonoid content was calculated using the following linear equation.

$$A = 0.01069\ C - 0.00163,\ r = 0.9998$$

Where:

A = is absorbance

C = is flavonoids content in µg.g-1

Estimation of saponin

Sources of Materials

The healthy and infected leaves, stems, inflorescence and seed of coriander *were* collected in the months of March at maturity stage of crops. The leaves, stem, inflorescence and seed were sun dried for seven days. The dried samples were then crushed with mortar and pestle before grinding into fine powder using a manual grinder.

Qualitative Determination of Saponin

The homogenous sample of each of the samples of the leaves, stem, inflorescence, and seeds were used for qualitative determination of saponin according to the methods described by Nyam *et al*[20]. A measured weight (5g) of the powdered sample was mixed with 50 ml of 20% aqueous ethanol solution in a flask. The mixture was heated with periodic agitation in water bath for90 minutes at 55ºC; it was then filtered through What man filter paper (No 42). The residue was extracted with 50 ml of 20% ethanol and both extract were poured together and the combined extract was reduced to about 40 ml at 90ºC and transferred to a separating funnel where 40 ml of diethyl ether was added and shaken vigorously. Re extraction by partitioning was done repeatedly until the aqueous layer become clear in colour. The saponins were extracted, with 60 ml of normal butanol. The combined extracts were washed with 5% aqueous sodium chloride (NaCl) solution and evaporated to dryness in a pre-weighed evaporation dish. It was dried at 60ºC in the oven and reweighed after cooling in desiccators. The process was repeated two more times to get an average. Saponin content was determined by difference and calculated as a percentage of the original sample as described by Harbone (1973).

$$\text{Saponin (\%)} = \frac{W2-W1}{\text{Weight of sample}} \times \frac{100}{1}$$

Where-
W1 = Weight of evaporating dish
W2 = Weight of evaporating dish + sample

Statistical Analysis

The quantitative data obtained were statistically analyzed by calculating the mean of three replicates followed by calculation of the Sum of Square, Variance, Standard Deviation and Standard error. The results were presented as mean + standard error.

RESULTS AND DISCUSSION

Symptomatology

The disease first appeared in the form of minute swelling on lower stem near soil level which later develops into gall. In young stage, galls were greenish yellow, soft and fleshy. But in later stages it becomes raised, grayish brown, elliptical, elongated in shape, hard and woody with the corky formation. Cracks were also observed on upper surface of plants. The numbers of galls on the plants were not definite; some pants have a large number of galls while other has only a few. The size of the gall ranged between 9-12 mm X 3-5 mm in diameter. The galls were distributed irregularly, crowded and random. At the flowering stage succulent shoots and branches have become twisted and distorted. Symptoms on leaves were observed in later stages of disease development when stem were covered with galls. Galls were first noticed on the veins of lower leaves and petioles in the form of minute brownish pustules. The abnormal growth on the petioles has been resembled with that of stem in texture and colour but it differs in size, being smaller. The outgrowth was observed inside of leaves around petioles. The infected leaves had changed yellowish in colour with minute elongated galls on mid vein and lateral veins. The leaf veins were found thick, fleshy, pallid and distorted. The minute, elongated with continuous ridges were observed on peduncles. They are brown in colour but smaller than found on stem. Sever infected peduncles have also showed malformation. Fruits of severely infected plants were found hypertrophied several times larger than normal fruits. The gall fruits were green in beginning which later turned brownish in colour. It was observed that either all fruits in umbel or only one or few fruits become infected and distorted.

The symptoms in the field are marked by appearance of minute swelling later developed in formed of galls on stem, leaves and fruits. The findings are supported by Srivastava[26]. Galls found on stem were greenish yellow, woody and rough scabby in appearance. In later stages of disease development, galls are converted into continuous ridges observed on infected branch let's showed a typical characteristic symptom known as 'goose neck' appearance[24]. It has been noticed that early stage of disease development, prominent brownish pustules appeared on the veins and petiole of lower leaves. Infected fruits become hypertrophied with the development of Chlamydospores. The external surface of fruit remains tender and glossy due to localized infection. Infected fruits become hypertrophied and several times larger than normal fruits. The tissues of diseased fruits are stimulated to grow the pathogen, unlike those in a normal fruits[23]. Akai[1] have reported that hypertrophy and hyperplasia completely change the normal arrangement of host cell because resulting body becomes bilaterally flattened and enlarged. The infected fruits become hypertrophied is might due to endosperm and embryo were lacking in fruit and increase in size by enlargement of fruit cells. While the external surface of the hypertrophied tissues remains tender and glossy green due to inhibition of sobering development[12].

Infestation source for spread of stem gall disease

Different infestation source of inoculums were mixed in sterilized soil before sowing of coriander seeds in pots. 30 days after sowing highest number of Chlamydospores were counted in total hypertrophied seeds inoculated pots. In other pots maximum number of chlamydospore was found in an

partially infected galled seeds (64.66/g soil) followed by infected plant debris (46.00/g soil) and previous year stem gall infected soil (42.00/g soil). None of the Chlamydospores were observed in healthy seeds (Table-1).

The primary inoculums of the pathogen are hypertrophied fruit gall mixed in an unclean seeds. Another source of inoculums is remains of plant debris. The infection becomes systemic in host before or during pre-flowering stage and infected parts become hypertrophied with development of Chlamydospores. The results are similar with Pavgi and Mukhopadhyay[23,24] and Lakra[15].

The mycelium and ascogenous cells of *Protomyces macrosporus* were found to present in infected tumour-like swellings on all herbaceous parts of the affected plants namely, stems, petioles, flower stalks and leaves. The findings are similar as reported by Bacigalova[3]. The thick septate mycelium was found within the intercellular space of tissues and concentrates mainly in vascular tissue. The ascogenous cells are formed intercalary on the intercellular mycelium. Parmasto & Parmasto[22] and Bacigalova, *et al*[4] are reported the septate mycelium is very thin invades into the intercellular space of leaf tissue and concentrates mainly in vascular tissue**.** Mature ascogenous cells are 36.5 μm long, 35 μm wide and three-layered4.7 μm thick cell wall with light yellowish to brown colour.

Number of chlamydospores around the plants

Perusal of Table (3) resulted that maximum average number of chlamydospore was found at 3 inch distance (141.70g soil^{-1}) and minimum at 2 inch distance (112.30 g soil^{-1}). In case of depth the highest average number of chlamydospores were recorded at soil surface (198.80 g soil^{-1}) followed by 1 inch (130.40 g soil^{-1}) and 2 inch depth (42.20 g soil^{1}). The variability in number of chlamydospore was significant between 3 inch and 2 inch distance at surface as well as 1inch depth and at par in 2 inch depth. The numbers of chlamydospore g soil^{-1} was higher on soil surface up to 3 inch distance and lesser on 1 and 2 inch depth at base of the plants. The Chlamydospores surviving in the soil depth remain protected for one to two years and are spread again by replanting the field. The higher chlamydospore load on the surface soil is due to continuous input of the Chlamydospores through intensive cultivation of the host crop[16,24].

Biochemical changes in healthy and infected plant parts of coriander

Perusal of data Table(2) revealed that total phenol content was highest in healthy plant parts (2.97-6.20µg/g)as compared with stem gall infected plant parts (2.53-5.58µg/g). The stem gall disease markedly decreased total phenol content in all parts of coriander. The reduction was 32.77% in stem, 14.80% in inflorescence, 10.00% in leaves and 8.44% in seeds. Total tannin content was significantly highest in leaves (3.29-3.92 µg/g) and lowest in inflorescence (2.31-2.79 µg/g) in both stem gall infected and healthy plants. Reduced tannin content was significant in all parts of infected plants. The reduction was markedly highest in inflorescence (17.20%) followed by stem (16.53%), leaves (16.07%) and seeds (15.07) over the healthy plant parts respectively. Healthy plants of coriander brought about significant enhancement of flavonoids content (0.98-1.47µg/g) as compared to infected plant parts (.77-1.07µg/g). Stem gall disease was markedly decreased the amount of flavonoids content (17.64-27.21%). The maximum reduction was observed in leaves (27.20%) and minimum in seeds (17.64%). Stem gall disease significantly decreased the saponin content (0.99-1.80%) as compared to healthy plants of coriander (1.93-2.16%). The maximum decrease was in inflorescence (48.70%) followed by leaves (23.61%), seed (14.50%) and stem (10.00%)

Plants are a rich source of thousands of secondary metabolites. These are low molecular weight compounds that are not essential for sustaining life but are crucial for survive of the organisms[29]. These compounds are phenol, tannins, flavonoids and saponins. Which lead to a range of defense responses in the host plants? In present study total phenol content was found highest in healthy plants and lowest in infected plants. Phenol

compounds may be confirming resistance to a disease by limiting the growth of the pathogen[13]. Stem gall infection was severe on stem hence reduction of phenol substances was found maximum in stem and minimum in seed. The finding was supported by Kuvalekar *et al*., Gogoi *et al*[10]., was observed an immediate accumulation of phenol following pathogenic attack in resistant varieties, whereas susceptible varieties did not accumulate significantly higher phenol substances.

Tannins content was significantly higher in leaves and lowest in inflorescence in both healthy and infected plants. Czech-Kozbowskee and Krywanasks[7] detected poly phenol oxides activity is higher in the infected resistant variety than in infected tissue of susceptible varieties. Tannins have been documented as antimicrobial compound because condensed tannins can inhibit basidiospore germination and also altered germ tube morphology of *C. perniciosa*[5,19]. Stem gall disease markedly decreased the flavonoid and saponin content in all infected parts, the maximum reduction was in leaves and inflorescence respectively. The high level of saponin in the root might be as results of the need to protect plant against soil borne pathogen attack. It has been also noted that many saponins are present in healthy plants in high concentration because of these antifungal properties[21]. Saponin presence in stem and stem bark might to be serving as natural defense against viral, bacterial and fungal infection[8].

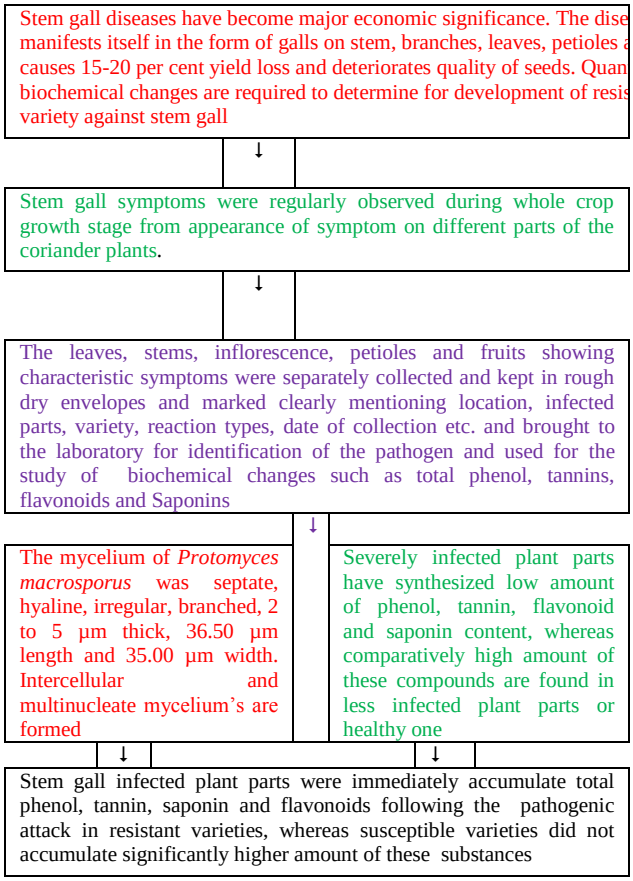

Table 1: Effect of different types of infestation source for the spread of stem gall in coriander

S. No.	Treatment	Plant Height (cm)	No. Of Branch's Plant^{-1}	Seed yield (g/plant)	Disease appearance	Avg. no.chlamy-dospores/g soil	Dry weight of straw(g)
1.	Infected plant debris	43.66	3.33	2.94	0	49.00	8.63
2.	Infected galled seed	31.00	2.66	2.87	0	64.66	6.25
3.	Infected plant seed without gall	41.33	2.66	3.1	0	0	7.27
4.	Pervious year stem gall infected field Soil	58.66	3.66	4	0	42	8.85
5.	Control (Sterilized soil only)	60.66	4.33	4.08	0	0	9.80
	SEm±	2.33	0.18	0.23	0	3.21	0.57
	CD at 5%	7.60	0.60	0.72	0	10.46	1.87

Table 2: Study of biochemical changes in healthy and infected plant parts of coriander

S. No.	Treatment	Total Phenol (µg/g)			Tannin (µg/g)			Flavonoids (µg/g)			Saponin (%)		
		Healthy	Infected	% decrease	Healthy	Infected	% decrease	Healthy	Infected	% decrease	Healthy	Infected	% decrease
1.	Leaves	6.20	5.58	10.00	3.92	3.29	16.07	1.47	1.07	27.20	2.16	1.65	23.61
2.	Stem	4.18	2.81	32.77	3.87	3.23	16.53	1.26	0.99	21.42	2.00	1.80	10.00
3.	Seed	4.5	4.12	8.44	3.78	3.21	15.07	1.19	0.98	17.64	2.00	1.71	14.50
4.	Inflorescence	2.97	2.53	14.80	2.79	2.31	17.20	0.98	0.77	21.42	1.93	0.99	48.70
5.	SEm±	0.12	0.26		0.16	0.19		0.14	0.08		0.08	0.11	
6.	CD (0.5%)	0.40	0.89		0.55	0.64		0.47	0.28		0.29	0.39	

Table 3: No of resting spores (chlamydospore) present in the soil around the coriander plant

S. No.	Location	Avg. number chlamydospore/g soil			
		Surface	1" depth	2'' depth	Mean
1.	Plant base	201.00	106.00	45.00	117.30
2.	2'' distance	176.33	117.66	43.00	112.30
3.	3'' distance	219.00	167.66	38.66	141.70
4.	Mean	198.80	130.40	42.20	123.76
Interaction					
		SEm±		CD at (5%)	
1	Distance	4.12		13.60	
2	Depth	3.89		12.19	
3	DistanceXDepth	6.25		21.12	

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