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Research Article

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Cytokinins effect on direct shoot bud regeneration from leaf segments of bitter cucumber (*Cucumis trigonus* Roxb.)

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ABSTRACT

Five different cytokinins effect on shoot regeneration from leaf segments culture of Cucumis trigonus was studied. Cytokinins 6-benzyladenine (BA), kinetin (kn), zeatin, dimethyl allyl amino purine (DAAP) and thidiazuron (TDZ) were used for direct shoot induction. Of the various cytokinins tested highest regenerative response was observed in leaf segments cultured in Murashige and Skoog's (MS) medium supplemented with 1.0 mg L^{-1} BA + 0.25 mg L^{-1} indole-3-acetic acid (IAA), where all the explants responded producing an average of 14.29 shoot buds per explant over a period of 6 weeks culture. Differentiation and elongation of shoot buds was achieved by sub-culturing them on MS augmented with 5.0 mg L^{-1}glutamine. Regenerated shoots were readily rooted in full and half strength MS medium and about 95% of the plantlets survived on transfer to outdoors.

Key words: *axenic leaves, Cucumis trigonus, cytokinins, glutamine, indole-3-acetic acid.*

INTRODUCTION

Cucumis trigonus Roxb. (Family: Cucurbitaceae) commonly known as bitter cucumber, is an wild relative of the cultivated cucumber *Cucumis sativus* L. It is distributed in India, Ceylon, Malaysia, Afghanistan, Persia and Northern Australia[1]. The plant is used for curing various ailments. The fruit pulp is bitter, acrid, thermogenic, anthelmintic, liver tonic, cardio tonic, appetizer, expectorant and intellect promoting. It is used in flatulence, leprosy, fever, jaundice, diabetes, cough, bronchitis, ascites, anaemia, constipation, other abdominal disorders and amentia[2]. The roots are used as purgative and the seeds are cooling and astringent. Chhattisgarh (India) farmers use the fruits as breakfast, which helps them to cure indigestion and helps them to work longer in the field[3]. The alcoholic extract of the plant reported to possess analgesic, anti-inflammatory[4] and diuretic[5] activity. The aqueous extract of its fruit was reported to show anti-diabetic activity in streptozotocin-induced diabetic rats[6]. The ethanol extract of the fruit has therapeutic and prophylactic value in isoproterenol induced myocardial infarction in male albino sprague dawely rats[7]. The plant has proteolytic and serine protease activity and has been reported to be used as a meat tenderizer[8,9,10]. Phytochemical investigations in *C.trigonus* have revealed the presence of four steroidal and triterpenic compounds such as stigma-7-en-3β-ol, stigma-7-en-3β-glucoside, alnusenone and alnusenol in its chloroform extract[11]. Cucurbitacin B was also isolated from the fruit[12].

Conservation of wild relatives of cultivated crops is important for modern agriculture as they provide a potentially useful genetic resource for breeding and improvement of related cultivated crops[13]. Use of biotechnological tools such as plant tissue culture for conservation of such species have been practised[14,15]. Among the various strategies *in vitro* propagation methods of adventitious regeneration is most preferred because of its suitability for Agrobacterium-mediated gene transfer experiments. While *C. trigonus* is a wild relative of present day cultivated cucumber, it is also a valuable medicinal plant. There

were no reports available on an *in vitro* culture of this species. The present work reports an efficient *in vitro* plant regeneration protocol for *C. trigonus* via adventitious shoot development from leaf explants cultured on medium containing different concentrations and combinations of various plant growth regulators (PGRs).

MATERIALS AND METHODS

Establishment of in vitro shoot cultures

In a preliminary experiment, leaf segments obtained from field grown plant resulted in heavy contamination and very poor shoot response was observed. Hence, it was decided to use axenic leaf explants. Mature nodal segments from 3 month old *C. trogonus* plant maintained in the nursery garden of CSIR-Institute of Minerals and Materials Technology, Bhubaneswar, India, was used to establish *in vitro* shoot cultures. The nodes were washed thoroughly under running tap water for 15 minutes followed by treatment with an aqueous solution of 5% (v/v) liquid detergent Labolene (Qualigens, India) and 0.1% (w/v) Bavistin (Bayer, India) for 10 min and rinsed with distilled water (5–6 changes). They were then surface disinfected with an aqueous solution of 0.1% (w/v) $HgCl_2$ (Hi-Media, Mumbai, India) for 5 min and rinsed 4–5 times with sterile double distilled water. The disinfected nodes were inoculated in 150 mm X 25 mm test tubes (Borosil, India; 1 node/tube) or Culture jars (Kasablanka; 5 nodes/jar) containing MS^{16} medium augmented with 3% w/v sucrose (Hi-Media, Mumbai), 0.8% (w/v) agar (Qualigens, Mumbai) and without growth regulators. The leaves from these regenerated shoots (Fig. 1A) were used as the tissue source for adventitious regeneration.

Shoot bud induction in axenic leaf tissue

In order to validate suitable cytokinin for effective shoot bud induction, cytokinin such as BA, kn, zeatin, DAAP and TDZ in the concentrations of 0.25 to 2.0 mg L^{-1} were employed along with MS basal medium. Further, to investigate the synergic effect of cytokinin and an auxin on shoot induction capacity cytokinin BA in the concentration of 0.25 to 2.0 mg L^{-1} and auxin IAA at 0.25 mg L^{-1} (selection of cytokinin and auxin concentration based on the response obtained in the preliminary experiments). All the growth regulators were bought from Sigma Chemical Co., St. Louis, USA. The pH of media was adjusted to 5.8 prior to gelling with 0.8% (w/v) agar and autoclaving at 121 ^{0}C and 104 kPa for 15 min. Depending on the requirement; media were dispensed into either 150 mm X 25 mm test tubes or in culture jars. Semi-mature leaves from nodal axenic cultures were excised and cut into small segments (0.5 cm^2) and transferred to various media composition (Table 1). All cultures were incubated in a culture room maintained at 55–60% relative humidity, 25 ± 2 ^{0}C under a 16 hour photoperiod of 40 µmol m^{-2} s^{-1} photon flux density provided by cool white fluorescent tubes.

Shoot elongation and in vitro rooting

After 4 weeks of culture period the responsive cultures were transferred to MS medium containing GA_3 (0.25 mg L^{-1}) and growth adjuvants such as 0.2% activated charcoal, glutamine (2, 4 and 5 mg L^{-1}) and arginine (2 and 4 mg L^{-1}). For *in vitro* rooting indole-3-butyric acid (IBA; 0.25 – 0.75 mg L^{-1}) or IAA (0.25 –0.75 mg L^{-1}) was incorporated in the agar gelled full and half-salt-strength MS basal medium.

Hardening and acclimatization

Three week old rooted plantlets were taken out from the culture vessels, washed thoroughly in running tap water to remove any remains of the nutrient-agar medium, and planted in polypots (5 cm diameter) and polybags containing autoclaved vermiculite saturated with 1/4th MS salt solutions. The plantlets were maintained inside a mist chamber set at 28 ± 2 ^{0}C, 85–90% relative humidity and 16 hours photoperiod of 50 µmol m^{-2} s^{-1} photon flux density provided with cool white fluorescent tubes (Philips, India) for 2 weeks. The acclimatized plants were then transferred to the polybags containing sand, soil and farmyard manure (1:1:1) and maintained in a greenhouse under intermittened misting and daylight conditions for 2 months. During this period humidity was gradually reduced to 65%. Well-hardened plants were subsequently transferred outdoors under full sun.

Statistical analysis

The experiments on the effect of plant growth regulators and growth adjuvants were conducted in a completely randomized design (CRD). To analyze the effect of selected growth regulators, singly or in

combination on adventitious shoot regeneration, each experiment was repeated 3 times. For shoot regeneration as well as root induction, each treatment consisted of 14 replicates. Data on percent response and the number of shoots per explant were determined after 6 weeks of culture and information regarding percent rooting and root number were determined after 3 weeks of culture. Data were analyzed with ANOVA for a completely randomized design (CRD). Duncan's multiple range test (DMRT)[17] was used to separate the means to determine significant effects.

RESULTS AND DISCUSSION

Shoot regeneration

The growth regulator type and concentration greatly influenced the adventitious regeneration capacity of the leaf segments. No regenerative response was observed in the leaf segments cultured in MS basal medium devoid of any growth regulators. Incorporation of cytokinins was proved beneficial and evoked varied responses (Table 1). Leaf segments cultured in 0.25 mg L^{-1} concentration of kn, zeatin and all the concentrations of DAAP failed to initiate shoot buds. BA at all the concentrations tested induced multiple adventitious buds from cultured leaf segments (Fig. 1B). The shoot buds in the responsive medium started to emerge from the surface of the leaf segment after 2-3 weeks of inoculation. The highest regenerative response was observed in the medium containing MS + BA (1.0 mg L^{-1}), where 100% cultures responded producing on an average 12.79 shoot buds per explant. BA induced adventitious shoot bud induction was also reported in *Cucumis melo*[18] and different genotypes of *Cucurbita pepo*[19]. The shoot bud production declined when the concentration of BA was increased beyond 1.0 mg L^{-1}. MS medium supplemented with kn and zeatin did not produce better response than any of the BA concentrations. The best response of kn and zeatin were observed on 1.0 mg L^{-1} where 2.43 and 1.43 shoot buds were produced per explant respectively (Table 1). Promotion of bud formation by cytokinin occurs in several plant species[20, 21, 22]. TDZ was proved effective at lower concentrations. At its higher concentration regeneration was low and was associated with callus formation. TDZ at 0.5 mg L^{-1} showed the best response, where 8.5 shoot buds were produced per explant. Variation in the activity of different cytokinins on adventitious regeneration from leaf segments of *C. trigonus* may be due to their differential uptake rate, as reported in other species such as Musa and Rhododendron[23]. Varied translocation rates to meristematic regions and metabolic processes, in which the cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds as reported by Tran and Trinh[24] and Kaminek[25].

Table 1. Effect of growth regulators on induction of adventitious shoots on leaf segments of *Cucumis trigonus*[1, 2 *]

MS + Growth regulator (mg L^{-1})					Days to shoot bud initiation	% response	Mean shoots/explant
BA	Kn	Zn	TDZ	IAA			
-	-	-	-	-	-	-	-
0.25	-	-	-	-	18-20	50	2.64 hij
0.5	-	-	-	-	16-18	64.28	6.79 cdefg
1.0	-	-	-	-	12-14	100	12.79 ab
2.0	-	-	-	-	14-16	85.71	8.07 cde
-	0.25	-	-	-	-	-	-
-	0.5	-	-	-	20-22	28.57	0.71 ij
-	1.0	-	-	-	18-20	42.85	2.43 hij
-	2.0	-	-	-	20-22	28.57	1.36 ij
-	-	0.25	-	-	-	-	-
-	-	0.5	-	-	20-22	21.42	0.71 ij
-	-	1.0	-	-	18-20	35.71	1.43 ij
-	-	2.0	-	-	20-22	21.42	0.36 j
-	-	-	0.25	-	14-16	71.42	5.93 cdefgh
-	-	-	0.5	-	12-14	71.42	8.5 cd
-	-	-	1.0	-	16-18	35.71	3.71 fghij
0.25	-	-	-	0.25	16-18	64.28	3.64 fghij
0.5	-	-	-	0.25	14-16	78.57	7.43 cdef
1.0	-	-	-	0.25	10-12	100	14.29 a
2.0	-	-	-	0.25	12-14	85.71	9.57 bc
3.0	-	-	-	0.25	16-18	57.14	4.5 efghi

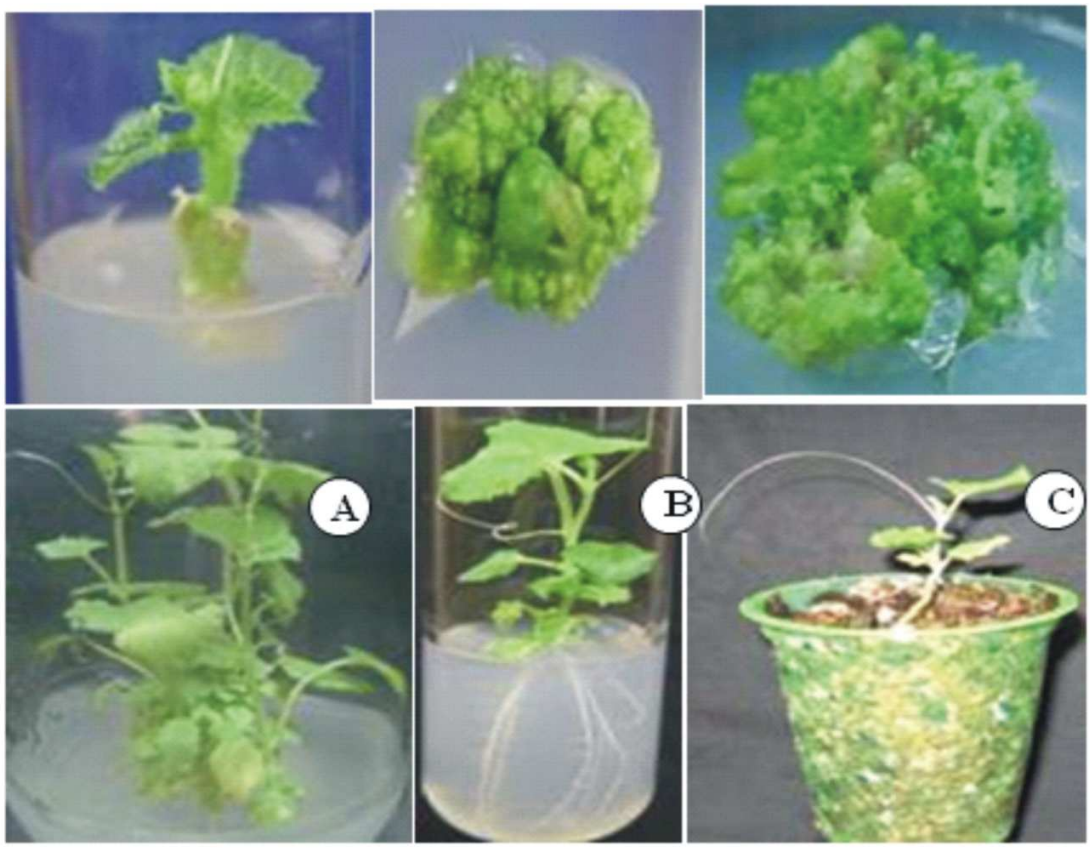
[1] Data pooled from three independent experiments each with 14 replicates per treatment.

[2] Data presented after 6 weeks of culture.

[*] Mean value within column followed by the same letter are not significantly different ($p \leq 0.05$; Duncan's Multiple Range Test)

A combined effect of cytokinin with auxin was also evaluated for multiple shoot induction. Addition of IAA along with BA to the basal medium enhanced the response significantly. Percent response and the number of shoots produced per explant were highest in MS basal medium supplemented with 1.0 mg L^{-1} BA + 0.25 mg L^{-1} IAA, where all the explants responded producing an average of 14.29 shoot buds per explant over a period of 6 weeks (Table 1 & Fig. 1C). Shoot regeneration was replaced by callus development in media with IAA at a high concentration i.e. 0.5 mg L^{-1}. The requirement for exogenous auxin and cytokinin in the process varies with the tissue system, apparently depending on the endogenous levels of the hormones present in the tissue[26]. During present investigations, direct organ formation was achieved on leaf segments on culture medium fortified with BA along with IAA (0.25 mg L^{-1}). Similar results were reported by Bairwa et al.[27] and Verma et al.[22]. The suitability of leaf segments for the micropropagation of *Cucumis* has previously been reported[18, 28, 29].

Fig. 1: Plant regeneration through adventitious shoot organogenesis in leaf explants of *C. trigonus*

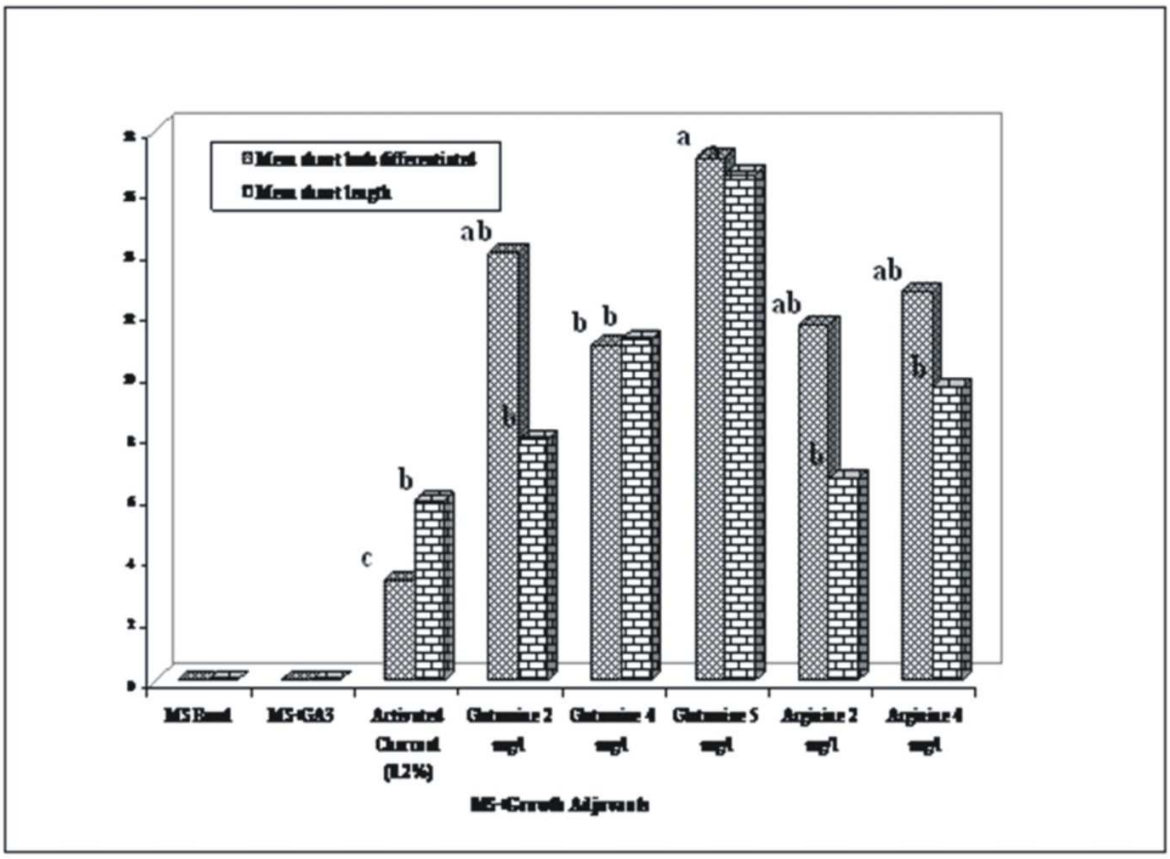


A- Shoot bud initiation from a cultured nodal segment on MS basal medium; **B** -Direct shoot regeneration on leaf segments after 2 wk in culture on MS basal medium supplemented with 1.0 mg/l BA; **C**- Direct shoot regeneration on leaf segments after 2 wk in culture on MS basal medium supplemented with 1.0 mg/l BA and 0.25 mg/l IAA; **D**- Leaf differentiation and shoot elongation in cultures on MS basal medium supplemented with 0.2% activated charcoal; **E**- *In vitro* shoot rooted on MS basal medium after 3 wk in culture; **F** -Acclimatized plants in polypot

Shoot differentiation and elongation

Explants with shoot buds initiated on MS medium containing growth regulators failed to differentiate and elongate on subcultured on to same medium or MS basal medium. In medium MS + GA_3 (0.25 mg L^{-1}) though the leaves got differentiated no elongation was observed. In MS + 0.2% activated charcoal medium, 50% of the cultures got differentiated and attained a height of 5.0 to 6.0 cm (Fig. 1D). About 92 % of the shoot buds cultured in MS + glutamine (5.0 mg L^{-1}) produced an average 17.71 elongated shoots/culture with an average height of 16.54 cm over 3 weeks time. The beneficial effect of glutamine on shoot bud induction was also described in adventitious regeneration from *Cucumis sativus* cotyledons[30]. Among the amino acids, glutamine at all the concentrations tested showed comparatively better differentiation and elongation of the shoot buds than arginine (Fig. 2).

Fig. 2: Effect of GA_3, activated charcoal, glutamine and argentine on shoot bud differentiation and elongation in *C. trigonus*



Bars with different letters are significantly different ($p \leq 0.05$; Duncan's New Multiple Range)

In vitro rooting

Root induction occurred in the cultured shoots on full and half strength MS basal media with no auxin (control). Among the full and half strength MS basal media, full strength was proved more efficient in root induction (Table 2). The roots produced in this media were comparatively longer and healthy. In MS basal medium all the shoots cultured produced 4.36 roots/shoot with an average length of 6.55 cm (Fig. 1E), where as in ½ MS only 78.57% shoots cultured, produced 4.79 roots/shoot with an average length of 4.55 cm. Full and half strength MS basal medium fortified with auxin showed varied response depending on the media strength, auxin type and concentration. Addition of auxin to the medium though produced more roots per shoot, the roots were comparatively shorter in length than those produced in control media. Of the different auxins investigated for rooting, IBA was proved superior to IAA in terms of root number and root length in both the half and full strength media (Table 2). Lower concentrations of auxins produced longer roots. Among full and half strength media augmented with IBA, half strength showed

better response in terms of root number (1/2 MS + IBA 1.0 mg L^{-1}; 6.5 roots/shoot) and root length (1/2 MS + IBA 0.25 mg L^{-1}; 3.9 cm). Among full and half strength media augmented with IAA, half strength showed better response in terms of root number (MS + IAA 1.0 mg L^{-1}; 4.86 roots/shoot) and in root length (1/2 MS + IAA 0.25 mg L^{-1}; 2.54 cm). The superior effect of IBA over other auxins such as IAA may be due to its slow movement, slow degradation and its localization near the site of application[31].

Hardening and acclimatization

In vitro hardening was a prerequisite for successful transfer of plants to soil. Rooted shoots transferred directly to pots without hardening showed wilting within 4-5 hr. But hardening of plantlets in green house with declining humidity gradient, helped in their better outdoor survival. Plantlets from all rooting media, showed better survival. It was evident that shoots with well developed roots have a greater survival tendency than those with shorter roots (Fig. 1F). Approximately 95% of the regenerated plants survived through the hardening process and successfully established outdoors.

Table 2. Effect of different concentrations of IBA and IAA on root induction in shoots of *C. trigonus* raised via adventitious regeneration. [1, 2 *]

MS +growth regulators (mg L^{-1})		Days taken to root initiation	% response	Mean roots/ shoot	Mean root length (cm)
IBA	IAA				
-	-	3-5	100	4.36 abcd	6.55 a
0.25		3-8	85.71	4.21 abcd	2.71 cdefg
0.5		3-4	92.85	3.79 bcd	2.36 cdefghi
0.75		4-8	92.85	5.71 abc	2.2 defghijk
1.0		4-6	92.85	6.07 ab	1.54 fghijklm
	0.25	3-16	92.85	2.86 d	1.06 hijklm
	0.5	8-16	92.85	3.36 bcd	0.53 lm
	0.75	3-6	100	4.07 abcd	0.45 m
	1.0	4-6	100	4.14 abcd	0.37 m
½ MS + growth regulators (mg L^{-1})					
IBA	IAA				
-	-	4-9	78.57	4.79 abcd	4.55 b
0.25		3-6	85.71	3.14 cd	3.9 bc
0.5		3-5	71.42	4.79 abcd	3.32 bcd
0.75		3-7	71.42	5.5 abcd	3.16 bcde
1.0		4-5	92.85	6.5 a	2.99 cdef
	0.25	4-6	100	3.5 bcd	2.54 cdefgh
	0.5	2-6	100	4.36 abcd	2.23 defghij
	0.75	4-6	100	4.57 abcd	2.04 defghijkl
	1.0	3-6	100	4.86 abcd	1.49 fghijklm

[1] Data pooled from three independent experiments each with 14 replicates per treatment.

[2] Data presented after 3 weeks of culture.

[*] Mean value within column followed by the same letter are not significantly different ($p \leq 0.05$; Duncan's Multiple Range Test)

CONCLUSIONS

The protocol outlined herein for a direct mode of adventitious *in vitro* multiplication of *C. trigonus* using leaf segments, can provide plant material for future pharmacological, physiological, and biochemical studies while giving a conservation measure for the plant.

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