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In vitro propagation of a rare succulent medicinal plant *Caralluma diffusa* (Wight) N.E.Br

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***Caralluma diffusa* (Wight) N.E.Br is rare and an endemic medicinally important plant belonging to the family Asclepiadaceae. A cost effective and efficient protocol has been described in the present work for large scale and rapid propagation through *in vitro* organogenesis using nodal explants cultured on Murashige and Skoog's (MS) media fortified with BAP, 2,4-D, NAA and Kn. The highest frequency (83.3%) of callus was observed in MS medium containing NAA (2mg/L) in combination with BAP (0.5mg/L) + Kn (0.25mg/L). Development of highest percentage of shoot organogenesis (82.4%) occurred when the calli were sub cultured in MS medium supplemented with combination of BAP + NAA + Kn (2.0 + 0.5 + 0.25mg/L). Multiple shoots were induced from the nodal segments cultured on medium containing BAP or Kn alone or in combination. Highest number of shoots (6.40±0.34) and maximum length of shoots (6.61±1.01cm) were obtained on MS medium containing BAP (2.5mg/L). These shoots were then cultured on half strength MS medium supplemented with various concentrations of IBA and NAA. Highest percentage of rooting (78.4%) and mean length of rootlets was observed on IBA (0.5mg/L) in combination with NAA (0.5mg/L). *In vitro* derived plantlets were hardened and acclimatized. About 98% of plantlets survived in field condition. The method described here can be successfully employed for large scale multiplication, long term *in vitro* conservation. This is the first report on indirect organogenesis of universally threatened cacti *C. diffusa*.**

Key words: *Caralluma diffusa*; indirect organogenesis; endemic; nodal explant.

Caralluma diffusa (Wight) N. E. Br is an endemic medicinal plant. After 160 years it was rediscovered from Southern Western Ghats of Coimbatore district, Tamil Nadu, India (Ramachandran *et al*., 2011). The Flora of Tamil Nadu states that *Caralluma diffusa* distribution is only from Coimbatore district in Tamil Nadu and ecological status is mentioned as rare and threatened species (Srinivasan, 1987). *Caralluma diffusa* is one of the endemic species found in Coimbatore (Henry *et al*., 1979). The genus *Caralluma* belongs to the family Asclepiadaceae which comprises of about 200 genera and 2500 species (Al-Yaha and Abdel-Sattar, 2002). In India, the genus is represented by 13 species and 5 varieties (Jagtap and Singh, 1999). Plants belonging to the genus *Caralluma* are

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normally leafless and succulent perennial herbs (Bailey, 1958). The species of *Caralluma* found in India are edible and form a part of the traditional medicinal system of the country. *Caralluma* species are commonly used in the treatment of rheumatism, diabetes, leprosy, antipyretic and anthelmintic, for tumor, fungal diseases, snake, scorpion bite and analgesic activity (Abdel-Sattar *et al*., 2007, The Wealth of India, 1992). *Caralluma umbellata* is used in Indian traditional medicine system for stomach disorder and abdominal pains (Vedavathy *et al*., 1997). *Caralluma adscendens* have significant analgesic (Tambe *et al*., 2010) and antimutagenic properties which promote immune system (Gowri and Chinnaswamy, 2011). Pregnane glycosides isolated from *Caralluma umbellata* have shown significant anti-inflammatory property (Sayanthan Ray *et al*., 2011). *Caralluma tuberculata* stem juice is used as bitter tonic, febrifuge, carminative, in rheumatism, is consumed as vegetable (Shinwari *et al*., 2006) and as antiparasitic medicine (Abdel Sattar *et al*., 2008). Increased commercialization has resulted overharvesting of these economically important medicinal plants, many of which have become threatened (Gustafsson, 2002). Asclepiadaceae plant species have poor seed germination and viability (Samydurai and Thangapandian, 2012). There has been over exploitation for commercial purpose and indiscriminate collection of *Caralluma diffusa* by the local civilians because of its therapeutic wonder and this has lead to severe biotic pressure. This species is naturally reproduced by clonal propagation, so minimum number of plants survived in the ecosystem. Propagation through seed is hampered by non-viability. In order to conserve this plant in wild, a regenerative protocol using micropropagation technique are developed.

Materials and methods

Source of plant material

Caralluma diffusa was collected from Madukkarai Hills of Coimbatore district, TamilNadu, India. They were grown in the aromatic medicinal garden of Kongunadu Arts and Science College, Coimbatore, TamilNadu, India. After 6 weeks young shoots of nodal segments were harvested and used as explant sources for micropropagation studies.

Explant sterilization

Collected explants were transferred to 250mL sterile conical flask and they were washed first under running tap water (20-30 minutes) and treated with 1% (v/v) Tween-20 for 10 minutes followed by repeated rinsing with distilled water. Subsequently they were treated (20 min) with 0.1% (w/v) carbendenzium (BASF, India). Further sterilization was done under aseptic environment in a laminar air-flow hood. Then the explants were surface sterilized with 60% (v/v) ethanol (1 minutes) and then by 0.01% (v/v) $HgCl_2$ (5 minutes). Finally, the explants were washed thoroughly (6-8 times) with sterile double distilled water. Further, explants were cut in to appropriate sizes (0.5-1 cm) and inoculated on MS medium.

Culture media and conditions

The explants were inoculated on MS basal (Murashige and Skoog's, 1962) medium supplemented with various auxins and cytokinins either individually or in combinations, containing 3% (w/v) sucrose. The pH was adjusted to 5.8 prior to the addition of 8 g/l agar and autoclaved at 121^0C for 20 minutes. Cultures were then incubated at 20 ± 2^0C with 16/8 hours photoperiod by cool white fluorescent tubes and 75-80% related humidity.

Callus Induction

For callus induction, nodal explants were cultured on MS medium containing different concentration and combinations of 6-benzylaminopurine (BAP; 0.5mg/L), Naphthalene acetic acid (NAA; 0.5-2.0mg/L), 2, 4-Dichlorophenoxy acetic acid (2, 4-D; 0.5-2.0mg/L) and Kinetin (Kn; 0.25-2.0mg/L). Data on callus induction efficiency were recorded after 4 weeks of culture. Callus induction frequency on different PGR'S in MS media formulation was calculated using the following equation.

$$\text{\% of frequency} = \frac{\text{No. of explants showing response}}{\text{Total number of explants inoculated}} \times 100$$

Shoot Regeneration

Callus obtained from the nodal explants after an incubation period of 30 days were sub cultured on MS medium augumented with BAP (0.5-2.0mg/L), NAA (0.25-1.5mg/L), Indole acetic acid (IAA; 0.5mg/L) and Kn (0.25mg/L) either alone or in combinations for rapid shoot organogenesis.

Multiple shoot proliferation

For direct adventitious shoot regeneration, nodal segments were used as explants, and inoculated on MS medium fortified with BAP (0.5-3.0 mg/L) separately and in combination with Kn (0.5-3.0mg/L).

Rooting

The regenerated shoots from the nodal explant derived callus, *in vitro* shoots were excised (3-7cm) and transferred to half strength MS medium fortified with NAA (0.5mg/L) combined with Indole-3-butyric acid (IBA; 0.5 to 2.5 mg/L).

Acclimatization

The rooted shoots were removed from culture bottles, washed with sterile double distilled water, to remove the traces of agar and planted in plastic pots containing mixture of soil, sand and sterilized vermicompost mixture in the ratio of 1:1:1. Once in three days half MS liquid medium without sucrose was added and kept in the culture room for 4 weeks after which they were transferred to the green house for acclimatization. The percentage of survival was recorded one month after transfer.

Statistical analysis

All the experiments were repeated four times with ten replicates per treatment, observations were made regularly and the data were carefully recorded. All the data were expressed as Mean ± SE. The statistical analysis of all the observations was carried out using one-way ANOVA ($P<0.05$) followed by Duncan's multiple range tests.

Results and Discussions

Callus induction

In our investigation callus induction was observed in MS media containing different concentrations and combinations of BAP, NAA, 2, 4-D and Kn. Within 15-23 days of incubation the nodal explants depending upon the concentration and combination of plant growth hormones induced callus initiation (Figure1 A). Wide range of variation in percentage of callus formation and physical appearance of callus was observed (Table 1). The highest percentage of callus induction (83.3%) was observed in MS medium containing BAP (0.5mg/L) + NAA (2.0mg/L) + Kn (0.25mg/L), and followed by 60.3 percentage in MS medium containing 2.0mgL^{-1} 2, 4-D and 0.25mgL^{-1} Kn (Table 1,Figure 1 B). Colour of the calli was mostly pale yellow to brown. The various combination of BAP, NAA, 2, 4-D and Kn produced compact and friable callus.

Multiple shoot proliferation

Nodal explants were cultured on MS medium fortified with different concentrations of BAP and Kn individually

and also in combinations for multiple shoot development and data have been presented in Table 2. Nodal explants cultured on medium with cytokinins showed gradual swelling after a week. Shoot regenerates directly without the intervention of callus formation. When BAP alone was used, better results were obtained only at certain optimum concentrations (0.5-2.5mg/L). The highest frequency of shoot formation was recorded (89.4%) in an optimum concentration of BAP (2.5 mgL^{-1}) with an average number of 6.40±0.34 shoots per node, with a mean length of 6.61±1.01 cm (Table 2).

Table 1: Effect of different concentrations and combinations of plant growth regulators on morphogenic response of callus induced from nodal segments explants of *C. diffusa* cultured on MS medium.

Plant growth regulators (mg/L)				Days to callus response	Colour	Texture of callus	Callus frequency
BAP	NAA	2,4-D	Kn				
0.5	0.5	-	0.25	17	PY	C	11.66±0.88^{g}
0.5	1.0	-	0.25	15	PY	F	39.66±0.76^{d}
0.5	1.5	-	0.25	15	PY	F	55±2.08^{c}
0.5	2.0	-	0.25	15	PY	F	83.3±1.45^{a}
-	-	0.5	0.25	21	B	C	10±0.57^{g}
-	-	1.0	0.25	20	B	C	23.66±1.38^{e}
-	-	1.5	0.25	19	B	C	41.33±0.71^{d}
-	-	2.0	0.25	19	B	C	60.3±1.8^{b}
-	-	-	0.75	23	B	C	2.4±0.67^{h}
-	-	-	1.5	23	B	C	8±1.9^{g}
-	-	-	2.0	23	B	C	19.6±1.76^{f}

PY – Pale yellow; B – Brown; F – Friable; C - Compact

Values represent mean ± standard error of 10 replicates per treatment in four times repeated. The Mean in a column followed by a same letters are not significantly ($P<0.05$) different according to Duncan's Multiple Range Test.

Table 2: Effect of different concentrations of cytokinins alone and in combination on multiple shoot proliferation from the nodal explants of *C. diffusa*

Cytokinins (mg/L)		Response (%)	No of shoots/explants (Mean ± SE)	Shoot length (cm) Mean ± SE
BAP	Kn			
0.5		38.01	1.71±0.12^{fg}	1.5±0.01^{ef}
1.0		57.5	2.62±0.09^{e}	2.30±0.34^{d}
1.5		74.1	3.96±0.19^{c}	4.6±0.17^{b}
2.0		81.9	5.21±0.26^{b}	4.9±0.12^{b}
2.5		89.4	6.40±0.34^{a}	6.61±1.01^{a}
3.0		43.05	1.80±0.06^{fg}	2.01±0.1^{d}
-	0.5	26.3	1.42±0.01^{ghi}	0.93±0.02^{gh}
-	1.0	44.6	1.77±0.19^{fg}	2.11±0.31^{d}
-	1.5	38.1	1.71±0.04^{fgh}	1.42±0.19^{ef}
-	2.0	30.3	1.49±0.02^{ghi}	117±0.06^{fg}
-	2.5	24.7	1.30±0.40^{hi}	0.93±0.05^{gh}
0.5	0.5	74.3	3.24±0.61^{d}	3.83±0.01^{c}
1.0	1.0	79.9	3.73±0.29^{c}	4.57±0.16^{b}
1.5	1.5	50.7	2.06±0.31^{f}	1.62±0.71^{e}
2.0	2.0	34.1	1.72±0.67^{fg}	0.9±0.02^{gh}
2.5	2.5	20.2	1.10±0.9^{i}	0.71±0.91^{i}
3.0	3.0	22.6	1.26±0.16^{i}	0.52±0.65^{i}

The Mean in a column followed by a same letters are not significantly ($P<0.05$) different according to Duncan's Multiple Range Test.

Table 3: The effect of various concentrations of PGR'S on shooting percentage, mean number and length of shoots explants of *C. diffusa* after 8 weeks of culture.

PGR				Shoot regeneration %	No. of shoots/explants Mean ± SE	Mean length of shoots/explants (cm)
BAP	NAA	IAA	Kn			
0.5	-	-	-	12.01	1.23 ± 0.03^{h}	0.57 ± 0.46^{l}
1.0	-	-	-	17.6	1.53 ± 0.08^{efgh}	1.66 ± 0.03^{h}
1.5	-	-	-	19.4	1.5 ± 0.19^{fgh}	1.86 ± 0.15^{gh}
2.0	-	-	-	51.72	2.16 ± 0.01^{d}	3.63 ± 0.06^{e}
2.5	-	-	-	76.6	3.16 ± 0.02^{c}	5.06 ± 0.14^{b}
3.0	-	-	-	31.09	1.63 ± 0.10^{efg}	2.1 ± 0.28^{g}
2	0.25	-	0.25	73.1	3.76 ± 0.42^{b}	4.86 ± 0.31^{b}
2	0.5	-	0.25	82.4	6.3 ± 0.28^{a}	6.2 ± 0.05^{a}
2	1.0	-	0.25	43.71	1.9 ± 0.05^{e}	2.9 ± 0.11^{f}
2	1.5	-	0.25	21.42	1.3 ± 0.11^{gh}	0.83 ± 0.05^{k}
1	-	0.5	-	16.9	1.7 ± 0.19^{ef}	1.42±0.17i
1.5	-	0.5	-	33.9	2.5 ± 0.1^{d}	2.1 ± 0.16^{g}
2	-	0.5	-	66.8	3.4 ± 0.23^{bc}	4 ± 0.09^{c}
0.75	-	-	0.25	13.5	1.4 ± 0.17^{fgh}	0.73 ± 0.04^{kl}
1.5	-	-	0.25	23.6	1.6 ± 0.31^{efg}	1.1 ± 0.03^{j}
2	-	-	0.25	63.3	2.4 ± 0.18^{d}	3.81 ± 0.51^{cd}

The Mean in a column followed by a same letters are not significantly (*P=0.05*) different according to Duncan's Multiple Range Test.

Table 4: Effect of various concentrations of IBA and NAA on rooting response from *in vitro* regenerated shoots of *C. diffusa*.

PGR		Rooting %	Mean No. of roots/shoot Mean ± SD	Mean length of roots (cm) ± SD	Root morphology
IBA	NAA				
0.5	0.5	78	7.6 ± 0.52^{a}	4.7 ± 0.1^{a}	Thin, long
1.0	0.5	46	5.6 ± 0.31^{b}	2.6 ± 0.4^{b}	Thin, long
1.5	0.5	35	3.3 ± 0.17^{c}	1.4 ± 0.73^{c}	Thin, long
2.0	0.5	28	2.1 ± 0.4^{d} Callus with root	0.92 ± 0.12^{d} Callus with root	Thick, short
2.5	0.5	24	1.8 ± 0.39^{e} Callus with root	0.63 ± 0.09^{de} Callus with root	Thick, short

The Mean in a column followed by a same letters are not significantly (*P<0.05*) different according to Duncan's Multiple Range Test.

Shoot and Root organogenesis

Shoot induction in nodal explants of callus were observed when it is sub cultured in MS medium with different concentrations of BAP, NAA, IAA and Kn either alone or in combination. Shoot initiations were observed after 20 days of inoculation and gradually the number of shoots per callus increased. The shooting response to auxins and cytokinins is shown in Table 3. The maximum percentage of shooting response (82.4%), number of shoots (6.3±0.28) and shoot length (6.2±0.05cm) was recorded in MS + 2mgL^{-1} BAP + 0.5mgL^{-1}NAA + 0.25mgL^{-1}Kn. Among

the various concentrations of BAP (0.5 – 3.0mgL^{-1}) about 0.5 to 2.5mgL^{-1} concentrations were showed positive responses.

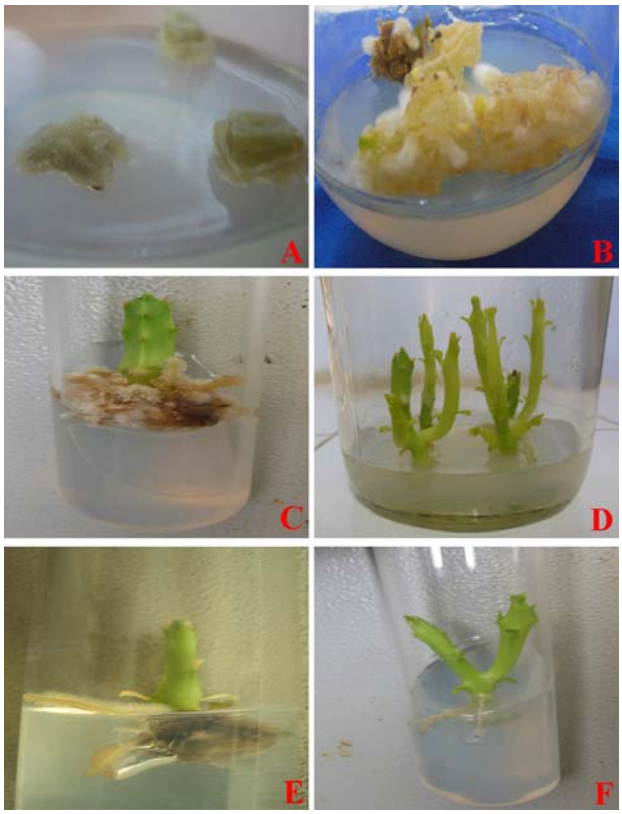

Figure. 1. ***In vitro*** **propagtion through nodal callus and multiple shoot proliferation of** ***C. diffusa***
(A) Callus initiation from nodal explants cultured on MS medium containing BAP + NAA + Kn; (B) Large and pale yellow callus formed on MS medium containing 0.5 mg/L of BAP + 2 mg/L NAA + 0.25mg/L Kn; (C) Shoot regeneration from subcultured callus on MS + 2mg/L BAP + 0.5mg/L NAA + 0.25mg/L Kn; (D) Multiple shoot proliferation after 30 days of inoculation on MS medium containing 2.5mg/L BAP; (E and F) Earlier stages induction of roots on regenerated shoots on half strength MS medium containing IBA and NAA.

IAA (0.5mg/L) in combination with BAP (1–2mg/L) has promoted moderate shoot regeneration than the other combination. In this investigation, the highest percentage (78.06%) of rooting was achieved in MS medium containing IBA (0.5mg/L) combined with 0.5mgL^{-1} IAA (Fig 2 D). The rooting frequency, number of roots per shoot and length of roots were recorded after 4 weeks of culture. The rooting response to both auxins treatment is shown in Table 4. Highest concentrations of IBA (2–2.5mg/L) combined with NAA (0.5mg/L) which induce callus formation along with rooting (Fig.2 A-B).

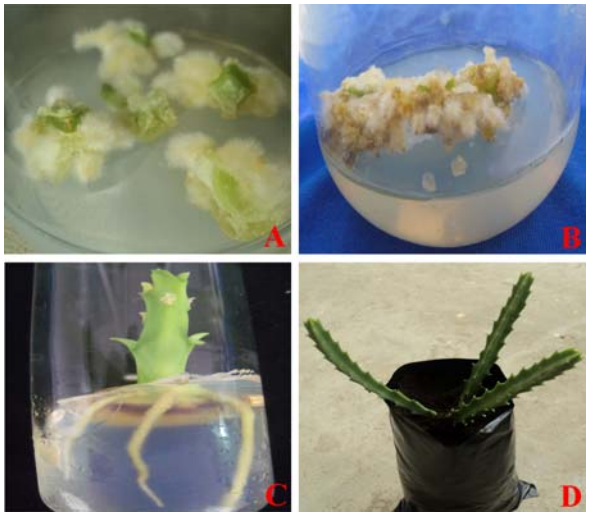

Figure. 2. (A and B) Induction of callus with rooting on regenerated shoots on half strength MS medium containing 2.0, 2.5mg/L of IBA + 0.5mg/L of NAA; (C) Extensive root growth on half strength MS medium containing 0.5 mg/L of IBA + 0.5 mg/L of NAA; (D) Acclimatized plantlet after four weeks.

Hardening

Finally the healthy regenerated plantlets were removed from the culture tubes and transferred to small plastic cups containing sand, fertile soil and vermicompost (1:1:1) under controlled

environment. After 4 weeks the plantlets were subsequently transferred to large pots and gradually acclimated to outdoor conditions. Over 98% of the plantlets were successfully acclimatized and *in vitro* grown plants did not show any detectable variations on their morphological characteristics when compared with parent.

Discussion

The influence of growth regulators on callus formation was investigated by using various concentrations and combinations of auxins with cytokinins (Table 1). Explants cultured with Kn alone showed poor callogenesis. The present investigation was directly coinciding with previous work were callus induction was found in NAA and 2, 4-D on Caralluma fimbriata (Rajaram *et al*., 2012). Jaberian *et al.,* (2012) also used BAP, 2, 4-D, NAA and TDZ for callus induction in *Falcaria vulgaris*. Similar favourable combained effect of auxins and cytokinins induce maximum callus frequency in *Leptadenia pyrotechnica* (Qureshi *et al*., 2012) *Aristolochia bracteata* (Sahaya Sathish *et al*., 2011), and *Oxystelma secamone* (Dharmendra *et al*., 2010). On the other hand the intermodal explants were cultured on MS medium supplemented with auxins and cytokinins induced callus in *Caralluma sarkariae* (Raja Sreelatha *et al*., 2009). However in the present investigation, a combination of NAA + BAP + Kn was proved as the most efficient medium composition for better callusing in *C.diffusa*. Response of multiple shoot proliferation was dependent on type and concentration of cytokinins supplements in the media. Between the two types of cytokinins, BAP was superior to that of Kn in terms of proliferation of shoots per node (Table 2). Similarly, multiple shoot formation achieved through direct regeneration from the nodal explants of *Boucerosia diffusa* cultured on MS medium fortified with cytokinins (Ramadevi *et al*., 2012). The efficiency of BAP in *in vitro* shooting has been successfully reported in other Asclepiadaceae species such as *Aristolochia bracteata* (Sahaya sathish *et al*., 2011) and *Marsdenia brunoniana* (Ugraiah *et al*., 2010). The supplementation of BAP either singly or in combination with Kn for shooting was also reported in many plant species (Meena *et al*., 2012; Akbas *et al*., 2011; Ashok *et al*., 2010). The pale yellow friable callus was transferred to shoot induction media. Highest frequency of Shootlets proliferations were obtained on MS medium augmented with BAP (2 mg/L) + NAA (0.5 mg/L) + Kn (0.25mg/L). Our findings are directly associated with previous work that shoot induction was found to be greater in BAP on *Dipteracanthus prostrates* (Robert *et al*., 2012), *Centella asiatica* (Karthikeyan *et al*., 2009), *Gynura procumbens* (Keng *et al*., 2009).There are few reports about multiple shoot induction by callus culture in *Cleome viscosa* (Anburaj *et al*., 2011), *Hemidesmus indicus* (Siddique and Bari, 2010), *Caralluma sarkariae* (Rajasreelatha *et al*., 2009), *Caralluma bhupenderiana* (Ugraiah *et al*., 2011). In our studies the best rooting rate happened on MS medium supplemented with 0.5mgL^{-1} IBA + 0.5mgL^{-1} NAA. In most species, efficient rooting was observed on medium containing auxins. NAA and IBA are most commonly used plant growth regulators for root induction (Bhojwani and Razdan, 1992). Similarly IBA has been used successfully to induce the highest rooting frequency in *Commiphora wightii* (Tejovathi *et al*., 2011), *Dregea volubilis* (Yogananth *et al*., 2011), *Ceropegia juncea* (Krishnareddy *et al*., 2011) and *Rubia cordifolia* (Radha *et al*., 2011).

Conclusion

An easy effective protocol is proposed for handling micropropagation of the endangered medicinal plant *Caralluma diffusa*, which is also a rapid successful technique for *ex-situ* conservation. As a part of severe biotic pressure, these plants can be grown and

cultivated in fields. This protocol's main application is to help minimize the pressure on wild populations and in conservation of the valuable flora of India.

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