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Effect of Explant Type in Development of *in vitro* Micropropagation Protocol of an Endangered Medicinal Plant: *Curcuma caesia* Roxb.

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Abstract: *Curcuma caesia* Roxb. belonging to family Zingiberaceae is an important traditional medicinal plant. The rhizome has been widely used as folklore medicine. Chhattisgarh state medicinal plant board has categorized it as an endangered plant. Turmeric/ginger is vegetatively propagated exclusively through underground rhizomes and multiplication rate is very low. Furthermore, susceptibility to diseases, specifically soft rot caused by *Pythium* species causes heavy losses. *In vitro* micropropagation technique can be useful for conservation and commercial exploitation of valuable secondary metabolites in medicinal plants. *In vitro* plant regeneration system depends upon many factors but the type of explants has been identified as one of the major factor. Thus, the present study was conducted using various parts of the plant viz. leaf, root, rhizome sections, mature bud of rhizome and sprouted bud of rhizome in different hormone concentration and combinations in MS medium. Only mature bud and sprouted bud from the rhizome responded while others did not show any sign of morphogenesis. Sprouted and mature bud showed best response in 4 mg L^{-1} BAP+100 mg L^{-1} ADS with mean 3.8±0.32 number of shoots and mean 3.28±0.42 cm length, similarly, 1±0.39 mean shoots and mean 1.17±0.48 cm length in mature bud. *In vitro* produced plants were easily established in soil with almost 100% survival and were morphologically similar to their parent plants. Thus, sprouted buds of the rhizome can be exploited for further micropropagation and conservation studies.

Key words: Micropropagation, endangered, explant, rhizome, folklore

INTRODUCTION

Curcuma caesia Roxb. commonly known as "Black turmeric" is an important medicinal plant belonging to family Zingiberaceae. The plant is a perennial herb, normally erect with 0.5 to 1.0 m height. It is divided into underground large rhizome and aerial shoot with leaves and flowers. The rhizome is tuberous with camphoraceous sweet odour, characteristic bluish colour and is the main propagating part of the plant. The leaves of the plant are broad, oblong, lanceolate and glabrous. Another morphological characteristic is presence of deep purple colour in the middle region of the lamina. The rhizome has a high economical importance due to its medicinal properties and commonly used as folklore medicine. It has been reported to contain curcuminoids, essential oil, flavonoids, phenols, different important amino acids, protein and high alkaloid content (Sarangthem and Haokip, 2010). This plant is found in Java, India and Myanmar (Sharma *et al*., 2011). Black Turmeric is native to North-East and Central India (Ravindran *et al*., 2007). It is also rarely found in Madhya Pradesh, Jharkhand, Chhattisgarh and Orissa (Mohanty *et al*., 2010). The members of Zingiberaceae reproduce exclusively by asexual clonal propagation of the rhizomes. The risk of transmittance of diseases from one generation to the next is great and *Pseudomonas solancearum* (bacterial wilt disease), *Fusarium oxysporum* F. sp. *Zingiberi* (fusarium yellows disease) and *Pythium* species (soft/root rot) are transmitted in this manner and lead to significant losses to growers (Ma and Gang, 2006). The rhizome of the plant is used in India for tantric sadhana and medication by tribal people, due to which it is over exploited and depleting faster. The National Medicinal Plant Board of India has listed it as crucially vulnerable species. Chhattisgarh state is known as "Herbal State" of India due to presence of wide varieties of medicinal plants. The Chhattisgarh medicinal plant board has identified 45 species along with *Curcuma caesia* as endangered species of the state. So, there is urgent need to conserve this natural resource by *in vitro* techniques. Thus, Micropropagation technique can be one of the efficient tools for *in vitro* propagation of medicinal plants for disease free plants, conservation and commercial

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exploitation of valuable plant-derived pharmaceuticals and it also assures the *ex situ* conservation of these plants. The present study was conducted to establish the *in vitro* micropropagation protocol using various parts of the plant viz. leaf sections, root sections, rhizome sections, mature bud of rhizome and sprouted bud of rhizome in different hormone concentration and combinations in (Murashige and Skoog, 1962) medium to standardize the hormone concentration and suitability of various explants for plantlet regeneration via callus formation or direct organogenesis.

MATERIALS AND METHODS

The rhizomes of *Curcuma caesia* were collected from medicinal garden of Indira Gandhi Agriculture University, Raipur (C.G.) and sown in the pots in shade house of School of Studies in Biotechnology, Pt. Ravi Shankar Shukla University. Raipur (C.G.). When the plant grew, leaf were taken for *in vitro* culturing, while some harvested rhizome were used for roots, mature buds and its slices were also cultured in MS media with varying concentration of different hormones and combinations for the callogenesis and further shoot regeneration and some rhizomes were planted in sand for sprouting of buds. All the explants were thoroughly washed with tap water to remove soil traces and washed with Tween 20, then were surface sterilized with 0.2% Bavistin for 15 min to half an hour and aseptically with 0.1-0.2% mercuric chloride solution for 15-30 min depending upon the explant type, then washed 5-6 times in sterile distilled water, each of the sterilized explants was cut into 2-3 mm pieces using sterile scalpel and inoculated onto the culture medium. All cultures were maintained in a growth chamber at a temperature of 25±2°C and 16 h photo period provided by white fluorescent tubes. All the data were collected after a month, experiments were repeated thrice and had ten replicates.

Statistical analysis: A complete randomized design was done in every experiment. Mean Standard deviation and standard errors were calculated. The data were subjected to one way analysis of variance (ANOVA) to assess treatment differences and interaction using the SPSS version 16 significance between means was tested by DMRT's Test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Establishment of aseptic cultures was a very difficult task in this plant, because the explants were taken from underground rhizomes. Nearly 60% of the cultures were found to be contaminated initially in the medium. However, once a healthy culture was established, there was no further contamination till the hardening of the plant. The present study was conducted using various parts of the plant viz. leaf, root, rhizome sections, mature and sprouted bud of rhizome (Fig. 1) in eight different hormone concentration, BAP (0, 0.25, 0.5, 1, 2, 4, 8 mg L^{-1}) and in combination with NAA (4 mg L^{-1} BAP and 2 mg L^{-1} NAA) in MS medium for shooting and MS+2, 4 D (2, 4, 6 mg L^{-1}) and combination (4 mg L^{-1} 2, 4 D and 2 mg L^{-1} BAP) for callogenesis (Table 1). Only mature bud (47.8%) and sprouted bud (100%) from the rhizome responded while others did not show any sign of morphogenesis. Sprouted bud showed best response in 4 mg L^{-1} BAP+100 mg L^{-1} ADS with the average 3.8±0.32 shoots and average shoot length 3.28±0.42 cm (Table 2). Within 1 month the whole plant was developed. Lower concentration of BAP resulted in single shoot but greater number of roots developed while in case of 2 and 4 mg L^{-1} BAP, multiple buds formed which give rise to multiple shooting while higher concentration of BAP (8 mg L^{-1}) resulted in single shoot development but of maximum length and lesser terminal branching (Fig. 2). Mature bud also showed best response in 4 mg L^{-1} BAP+100 mg L^{-1} ADS with the average 1±0.39 shoots and average shoot

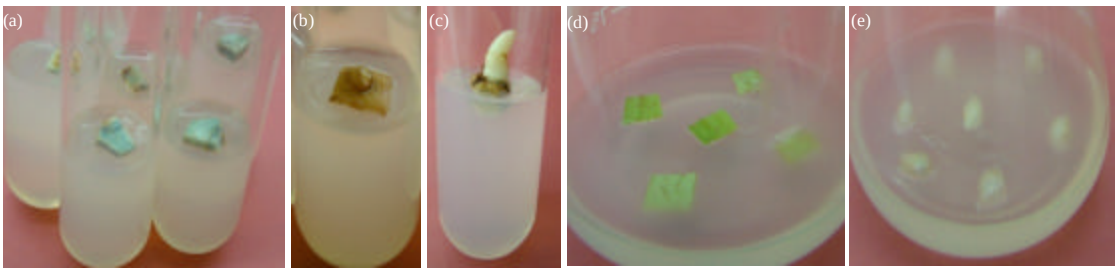

Fig. 1(a-e): Different types of explants used for *in vitro* response in MS medium (a) Rhizome sections, (b) Mature bud, (c) Sprouted bud, (d) Leaf sections and (e) Root sections

Table 1: Response of explants types in different hormonal concentration in MS medium

Type of explant	Hormone conc. (mg L^{-1})	Response (%)	Type of response	Remarks
Rhizome sections	MS+0 BAP	-	-	-
	MS+0.5 BAP			
	MS+1BAP			
	MS+2 BAP			
	MS+4 BAP			
	MS+4 BAP+2 NAA			
	MS+2 2,4,D			
	MS+4 BAP+2 2,4,D			
Root sections	MS+2 2, 4D	-	-	-
	MS+4 2, 4D			
	MS+6 2, 4D			
	MS+4 BAP+2 NAA			
Mature buds	MS+0 BAP	47.8	Bud break and shooting	Bud break was found in MS+4 and 8 mg L^{-1} BAP and subsequent shooting
	MS+0.25BAP			
	MS+0.5 BAP			
	MS+1BAP			
	MS+2 BAP			
	MS+4 BAP			
	MS+8 BAP			
	MS+4 BAP+2 NAA			
Leaf sections	MS+2 2, 4D	-	-	-
	MS+4 2, 4D			
	MS+6 2, 4D			
	MS+4 BAP+2 NAA			
Sprouted bud of the rhizome	MS+0BAP	100	Shooting	Shoots formed in all, irrespective of the hormone concentration. But varied in type of response
	MS+0.25BAP			
	MS+0.5 BAP			
	MS+1BAP			
	MS+2 BAP			
	MS+4 BAP			
	MS+8 BAP			
	MS+4 BAP+2 NAA			

Table 2: Response of sprouted bud in different hormonal concentration

Explant type	Hormonal conc. (mg L^{-1})	Mean No. of shoots±S.E.	Mean shoot length±S.E	Mean root No.±S.E	Mean root length±S.E.
Sprouted rhizome bud	MS+0 BAP	1.0 ± 0.00^{a}	2.15 ± 0.11^{a}	0.6 ± 0.16^{a}	0.20 ± 0.06^{a}
	MS+0.25 BAP	1.2 ± 0.13^{ab}	2.30 ± 0.12^{ab}	0.7 ± 0.12^{a}	0.25 ± 0.00^{a}
	MS+0.5 BAP	1.3 ± 0.15^{ab}	2.80 ± 0.18^{abc}	0.6 ± 0.16^{a}	0.23 ± 0.07^{a}
	MS+1 BAP	1.7 ± 0.15^{bc}	2.60 ± 0.16^{abc}	0.7 ± 0.15^{a}	0.35 ± 0.07^{a}
	MS+2 BAP	2.0 ± 0.29^{c}	2.96 ± 0.12^{bc}	1.1 ± 0.1^{ab}	0.45 ± 0.04^{a}
	MS+4 BAP	3.8 ± 0.32^{d}	3.28 ± 0.42^{c}	3.1 ± 0.27^{d}	1.70 ± 0.15^{b}
	MS+8 BAP	1.7 ± 0.15^{bc}	4.70 ± 0.42^{d}	1.4 ± 0.22^{bc}	0.41 ± 0.03^{a}
	MS+4 BAP+2 NAA	1.6 ± 0.16^{abc}	3.20 ± 0.24^{c}	1.9 ± 0.23^{c}	0.45 ± 0.04^{a}

Data (Mean±S.E.) were collected every 4 weeks from three experiments, each with 10 replicas, Means followed by the same letters in each column are not significantly different at $p<0.05$ according to Duncan's Multiple Range Test

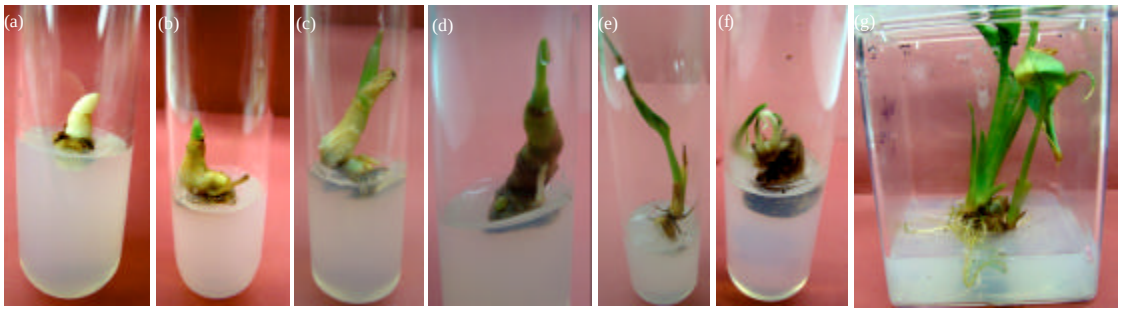

Fig. 2(a-g): Developmental stages of sprouted bud in MS+4 mg L^{-1} BAP+100 ADS, (a) Sprouted bud, (b) Bud turns green in one week, (c) 15 days after inoculation, (d) Roots induction, (e) Leaf induction, (f) Multiple bud induction and (g) Fully developed plant

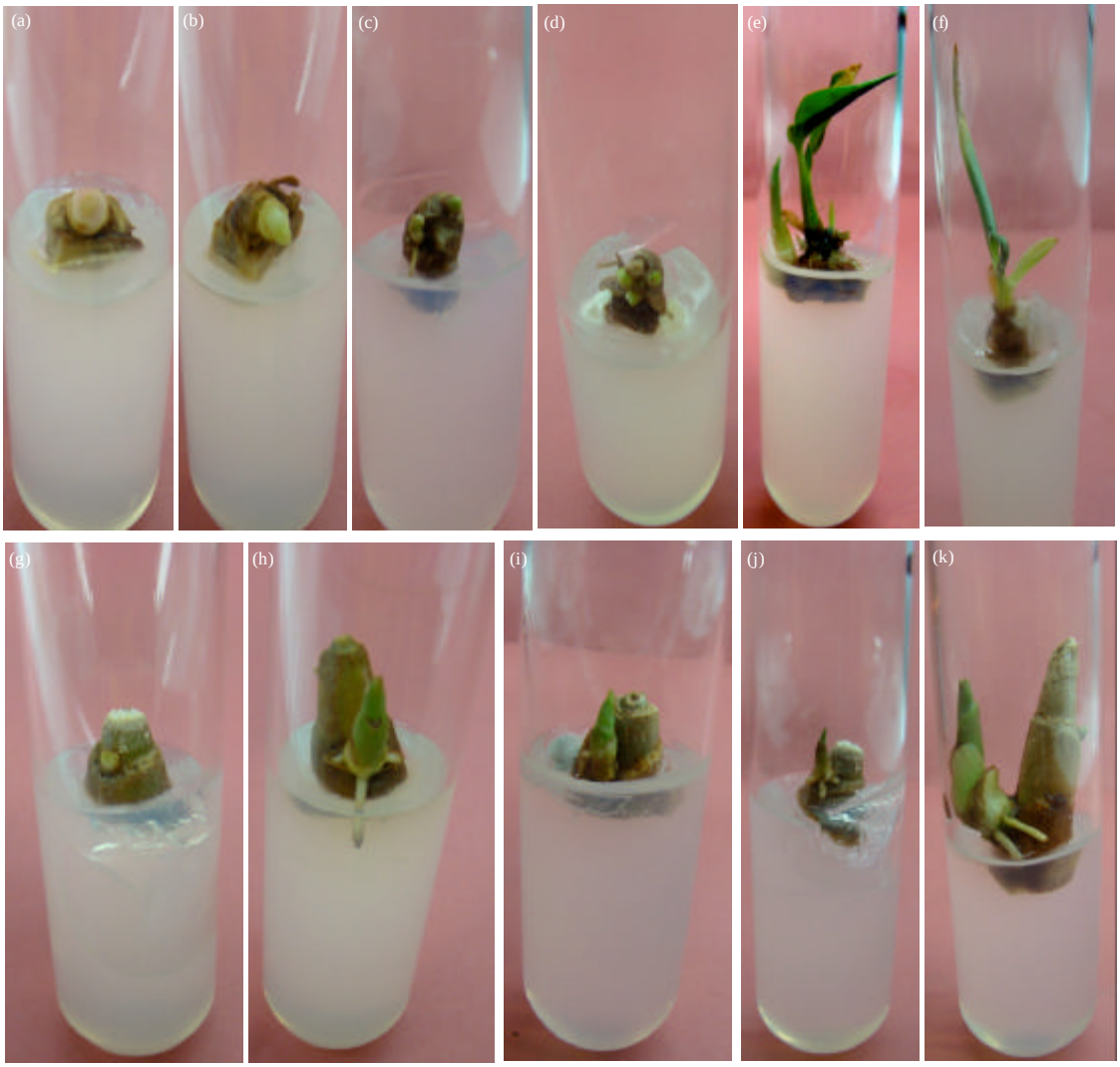

Fig. 3(a-k): Developmental stages of mature bud in MS+4 mg L^{-1} BAP+100 ADS, (a) Mature bud, (b) White bud turns green in 1 week, (c-d) Multiple bud induction, (e-f) 1 month old plant and (g-k) Mature bud dried, but in march new bud arises and roots and shoots simultaneously developed in the culture

Table 3: Response of mature bud in different hormonal concentration

Explant type	Hormonal conc. (mg L^{-1})	Mean no. of shoots±S.E.	Mean shoot length±S.E	Mean root no.±S.E	Mean root length±S.E.
Mature bud	MS+0 BAP	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
	MS+0.25 BAP	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
	MS+0.5 BAP	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
	MS+1 BAP	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
	MS+2 BAP	0±0.0^{a}	0±0^{a}	0±0^{a}	0±0^{ba}
	MS+4 BAP	1±0.39^{b}	1.17±0.48^{b}	1.9±0.64^{b}	0.7±0.15^{b}
	MS+8 BAP	0.6±0.22^{b}	1.55±0.53^{b}	0.8±0.32^{a}	0.40±0.02^{b}
	MS+4 BAP+2 NAA	0.8±0.16^{b}	1.12±0.39^{b}	1.65±0.23^{b}	0.38±0.01^{b}

Data (Mean±S.E.) were collected every 4 weeks from three experiments, each with 10 replicas, Means followed by the same letters in each column are not significantly different at $p<0.05$ according to Duncan's multiple range test

length 1.17±0.48 cm (Table 3) (Fig. 3). The mature buds were inoculated in the month of October-November (rhizomes are normally harvested), before the dormancy starts but bud break and shooting started in the month of March (Fig. 3g-k), the cultures were regularly sub-cultured in fresh medium and maintained in the lab. They showed

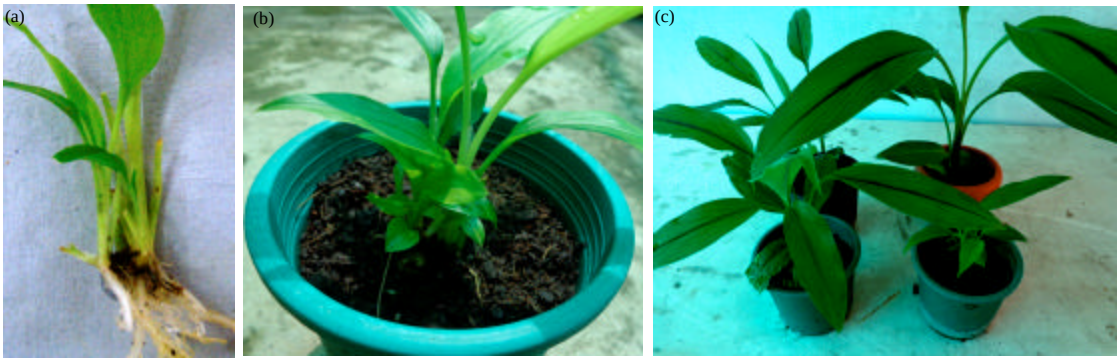

Fig. 4(a-c): Hardening of the *in vitro* generated plants, (a) Profusely roots developed, (b) Freshly hardened plant do not show characteristic purple colour in the midrib and (c) Well hardened plants morphologically similar to their parent plants

very late response and shoots were stunted, but when hardened grew normally, alternatively in the form of mature bud, it could be conserved throughout the year. Newly formed shoots were excised aseptically from the parent plant and transferred in the fresh medium with same concentration as the rooting was initiated in the similar media as used for shooting with average of 3.1±0.27 roots and average root length 0.7±0.15 cm in sprouted bud and in mature bud 1.9±0.64 roots and average root length 0.7±0.15 cm. A peculiar feature was observed in this plant as roots and shoots both developed simultaneously. *In vitro* produced plants were easily established in soil with almost 100% survival and were morphologically similar to their parent plant. The plants grown *in vitro* did not showed the characteristic purple colouration in the midrib region of leaves but on transfer to the cocopeat and then to soil developed normal pigmentation (Fig. 4).

In *Zingiberaceae*, different plant organs have been used as explants, the most successful for direct regeneration are the rhizome buds, but culturing efficiency of buds from "mature rhizomes" (rhizomes obtained after plants are harvested at the end of the season) and "immature rhizomes" (rhizomes obtained while plants are still growing in the field) have not been much studied (Ahmad *et al*., 2011). *Curcuma caesia* is a perennial herb, the rhizome remains alive underground throughout the year but the leaves emerges in March-April (onset of summer) and droops and dries out in the month of October-November (onset of winter) and sprouting in the rhizome also occurs in summer-rainy season. Thus, *in vitro* regeneration from aerial parts and sprouted bud of the plant becomes season specific. The development of *in vitro* micropropagation protocol depends upon various factors such as explant type, composition of culture media, exogenous supply of hormone and other cultural conditions. The type of explants has been identified as one of the major factors affecting the *in vitro* response in crop plants e.g. hypocotyl in case of linseed has been found superior than cotyledon and leaf irrespective of three genotypes used (Chandel *et al.,* 2011). The first report on *in vitro* regeneration and conservation in *C. caesia* was reported by Balachandran *et al*. (1990), using rhizome sprouted bud in MS medium. MS medium with BAP (3 mg L^{-1}) was found to be best combination. He also reported that 50% of the cultures were contaminated initially. Then Tyagi *et al*. (2004) developed *in vitro* plant regeneration and genotype conservation in eight varieties of *Curcuma*. They found 11.2 or 22.2 μM BAP as the best concentration of hormone for shoot regeneration in *C. caesia*. Bharalee *et al*. (2005) reported *in vitro* clonal propagation of *Curcuma caesia* Roxb. and *Curcuma zedoaria* Rosc. from rhizome bud explants and MS+BAP (4.0 mg L^{-1})+NAA (1.5 mg L^{-1}) was found to be most suitable for multiple shoot regeneration in *C. caesia*. Mohanty *et al*. (2010) used sprouted rhizome buds of *Curcuma caesia* as explants on MS media with various combination of BAP, IAA, NAA and kinetin. The percentage of explants forming shoots was highest in MS+BAP (3 mg L^{-1})+IAA (0.5 mg L^{-1}). All the previous authors have reported regeneration through sprouted bud of the rhizome, as in our studies also mature and sprouted bud both responded in cultural conditions. There are also some authors which have used different explants in the plants belonging to family Zingiberaceae and found positive results. Malabadi *et al*. (2005) reported the highest percentage of shoot regeneration of *Costus speciosus* using thin rhizome sections cultured on

B5 basal medium with triacontanol (TRIA). *In vitro* regeneration of ginger using leaf, shoot tip and root explants was reported by Sultana *et al*. (2009) using different concentration of Dicamba and 2, 4 D. Leaf explant gave best result over shoot tip and root explant. These results are not in accordance with our results as leaf, root and rhizome sections did not responded, which can also be because of different hormone, medium and cultural conditions.

CONCLUSION

In conclusion, the sprouted buds of the rhizome can be exploited for further micropropagation and conservation studies in *Curcuma caesia*. Standardization of explant type in development of *in vitro* regeneration system in this plants holds relevance as it is an endangered plant and lesser amount of explants are available for studies so having standardized the explant type, will help us in making effective efforts towards one type of explant and achieve goal for micropropagation and conservation of this plant species.

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