**MICROPROPAGATION VIA EMBRYOGENESIS TECHNIQUES FOR REFORESTATION OF VALUABLE TEAK (TECTONA GRANDIS LINN.F)**

**ABSTRACT**

**Introduction:** Teak is a valuable woody timber trees in industry.

**Aim**:It has been restored and micropropagated for reforestation

**Methods**: by using plant cell technology. The basic medium MS (MS + Ascorbate (5 mg/l) + vitamin B5 (5 mg/l) + glutamine (100 mg/l) + CW (10%) favored for embryogenesis culture was used for whole experiments

**Results:**Young shoot tip, leaves, and roots were used to initiated cultures. Callus cells were initiated on MS + BA (1 mg/l) + Ki (1 mg/l) + NAA (0,5 mg/l) after 30 days. Callus cells were differentiated to pro-embryogenic callus cells after subcultured on semi-solid basic medium MS + 2.4D (1 mg/l) after 30 days. Pro-embryonic callus cells sourced from shoot tip were seperated on liquid basic medium MS + BA (0,5 mg/l) + TDZ (0,2 mg/l) + NAA (0.2 mg/l) after 14 days. Pro-embryogenic callus cells were multiplied on liquid basic medium MS + BA (0,5 mg/l) + TDZ (0,2 mg/l) + TDZ (0.2 mg/l) after 30 days. Pro-embryogenic callus cells were differentiated into embryogenic cells on layering basic medium MS + BA (0,5 mg/l) + TDZ (0,2 mg/l) + NAA (0.2 mg/l) after 30 days. The conditioned basic medium MS + BA (0,5 mg/l) + TDZ (0.2 mg/l) + NAA (0,1 mg/l) was required for stimulation process before transfering embryogenic cells to regeneration media. Shoots were regenerated on medium MS + BA (0,5 mg/l) + TDZ (0.2 mg/l) + NAA (0,1 mg/l). A system for regeneration of teak somatic embryogenesis culture was established. Plantlets from embryos were used as materials to avoid degeneration for microprpagation and reforestation.

**Conclusion*:***A pilot scale for avoid of degeneration, regeneration and micro propagation system using somatic embryo technology has been established

*Keywords:* Teak (Tectona grandis Linn.F.), layering, callus cluster, cell suspension, differentiation, pro-embryogenic callus cells

**1. INTRODUCTION**

Teak (*Tectona grandis* Linn.f.) is an important valuable timber species (Monteuuis, 2021) because of its timber value (Rijuta and Sharon, 1996; [Iwao Noda](https://www.scirp.org/journal/articles?searchcode=Iwao+Noda&searchfield=authors&page=1) and [Woraphun Himmapan](https://www.scirp.org/journal/articles?searchcode=Woraphun+Himmapan&searchfield=authors&page=1), 2014). However, teak trees grow slowly with a harvesting cycle of 15-20 years (Kamal, *et. at.,* 2022), and the seed germination rate is low ([Palupi](https://www.journals.uchicago.edu/doi/abs/10.1086/297437)  and [Owens](https://www.journals.uchicago.edu/doi/abs/10.1086/297437), 1997; Callister and Collins, 2008; Baghel *et. al.,* 2008; Omokhua and Alex, 2015; Akram and Aftab, 2016; Mishra *et. al.,* 2018; Venkatesan *et. al.,* 2022; Venkatesan *et. al.,* 2024; Kurra *et. al.,* 2024; Amadi *et. al.,* 2024)

Tissue culture is a method of rapid propagation of teak trees that has been used in many countries in tropical Asia such as Myanmar, Thailand, Indonesia, Malaysia (Kaosar-ard and Apavatjrut, 1988). There are many studies on micropropagation (Singh *et. al.,* 2005; Akram and Aftab, 2009; Senthilkumar, 2015; Singh and Mishra, 2016; Hardiwinoto *et. al.,* 2021; Ruane *et. al.,* 2023).

By stem or shoot tip culture (Bonga and Aderkas, 1992; Goh and Monteuuis, 1995; Aftab and Preece, 2007; Akram and Aftab, 2008; Baghel *et. al.,* 2008; Santo *et. al.,* 2014; Sreedevi and Damodharam, 2015; Tambarussi *et. al.,* 2017; Yahya *et. al.,* 2020; Stephane *et. al.,* 2023; Sulaiman *et. al.,* 2023). Organogenesis by culturing young and old leaves (Graudal *et al.,* 1999; Rajendra *et. al.,* 2019), Direct and indirect somatic embryogenesis (Rijuta and Sharon, 1996; Lim Sooi Ping, 2008; Galeano *et. al.,* 2014; Nathalang and Bodhipadma, 2015; Akram and Aftab, 2016; Mishra *et. al.,* 2018).

Improving teak varieties by biotechnology with the aim of creating new varieties with advantages such as short growth time, straight trunk, few branches, slow flowering, wood color, disease resistance... the first barrier is the somatic embryo regeneration culture technique. This report studies the process of somatic embryogenesis and regeneration of teak trees.

**2. MATERIALS AND METHODS**

**2.1 Materials**

**Samples:** The culture sample is in vitro teak tree, imported from Myanmar.

**Media:** The basic culture medium is Murashige and Skoog (1962) and Lloyd and McCown (WPM, 1981). The substances added to the culture medium are: BA (6-Benzylaminopurine), Kinetin (6-Furfurylaminopurine), NAA (α-Naphthalene acetic acid), IBA (α-Indol butyric acid), TDZ (Thidiazurone), Ascorbat (5 mg/l), vitamin B5 (5 mg/l), glutamine (100 mg/l), coconut water 10 % (CW).

**Culture conditions:** room temperature 28+2oC, humidity 65%, lighting time 8 hours/day, lighting intensity 33.6 µmol/m2/s. To create cell suspension by cultivation cell clusters in erlenmeyer glass 300 ml, contain 65 ml media, speed at 100 rpm, incubation time 30 days

**2.2 Experimental designs**

**Callus culture initiation:**Culture samples are shoot tips, leaves, roots. Basic medium MS + Ascorbate (5 mg/l) + vitamin B5 (5 mg/l) + glutamine (100 mg/l) + CW (10%). Incubation time 30 days. Experimental variants:

A1, A5, A9: BA (1 mg/l) + Kinetin (1 mg/l) + NAA (0.5 mg/l)

A2, A6, A10: BA (1 mg/l)

A3, A7, A11: BA (1 mg/l) + Kinetin (1 mg/l)

A4, A8, A12: BA (1 mg/l) + Kinetin (1 mg/l) + NAA (0.5 mg/l)

**Culturing embryogenic callus cells on agar medium:** Culture samples are callus cells sourced from shoot tips, leaves, roots. Basic medium MS + Ascorbate (5 mg/l) + vitamin B5 (5 mg/l) + glutamine (100 mg/l) + CW (10%). Incubation time 30 days. Experimental variants:

B1: (0.5 mg/l),

B2: (1 mg/l),

B3: (2 mg/l)

**Rapid multiplication of embryogenic callus cells on liquid medium:**Culture samples are callus cluster sourced from shoot tips. Its has mean of 200 mg/cluster and cultivation in liquid media. Container is erlenmayer glass 300 ml, contained 65 ml media, placed on shaker set up 100 rpm. Basic medium MS + Ascorbate (5 mg/l) + vitamin B5 (5 mg/l) + glutamine (100 mg/l) + CW (10%). Incubation time 30 days. Experimental variants:

C1: BA (0.5 mg/l) + Kinetin (0.5 mg/l) + NAA (0.2 mg/l)

C2: BA (0.5 mg/l)

C3: 2.4D (0.3 mg/l)

C4: BA (0.5 mg/l) + TDZ (0.2 mg/l) + NAA (0.2 mg/l)

On the basic culture medium supplemented with BA (0.5 mg/l) + TDZ (0.2 mg/l) + NAA (0.2 mg/l). Initial biomass study is carried out in suitable culture. Incubation time 30 days. Experimental variants:

C5: (624 mg/cluster),

C6: (1248 mg/cluster),

C7: (1872 mg/cluster)

In experiment, Erlenmayer glass 300 ml was used, containing callus cluster and 65 ml media. Its placed on shaker with rotate speed 100 rpm

**Layering pro-embryogenic cells on agar medium:**Culture samples are callus cluster sourced from shoot tips. Cultured in liquid medium, two types of samples were collected: cell clusters and cell suspensions. Both types of samples were spread on embryogenesis medium to perform embryo. Basic medium MS + ascorbate (5 mg/l) + vitamin B5 (5 mg/l) + glutamine (100 mg/l) + CW (10%). Incubation time 30 days. Experimental variants:

D1: BA (0.5 mg/l) + Kinetin (0.5 mg/l) + NAA (0.2 mg/l) +

D2: BA (0.5 mg/l)

D3: 2.4D (0.3 mg/l)

D4: BA (0.5 mg/l) + TDZ (0.2 mg/l) + NAA (0.2 mg/l) +

A necessary period of incubation on embryo regeneration stimulating medium is required. Both types of samples were spread on experimental medium. Incubation time 30 days.

D5: BA (0.5 mg/l) + NAA (0.2 mg/l)

D6: BA (0.5 mg/l)

D7: BA (0.5 mg/l) + TDZ (0.2 mg/l) + NAA (0.1 mg/l)

**Somatic embryo regeneration:** Culture samples are callus cluster sourced from shoot tips. Callus clusters differentiated to embryo were used. Basic medium MS + ascorbate (5 mg/l) + vitamin B5 (5 mg/l) + glutamine (100 mg/l) + CW (10%). Incubation time 120 days. Experimental variants:

E0: non-Plant growth regulator

E1: TDZ (0.2 mg/l) +

E2: TDZ (0.5 mg/l) +

E3: TDZ (0.5 mg/l) + NAA (0.2 mg/l) +

E4: CW (10%)

E5: BA (1 mg/l) + TDZ (0.5 mg/l) + CW (10%)

E6: BA (1 mg/l) + TDZ (1 mg/l) + CW (10%)

E7: BA (1 mg/l) + TDZ (1 mg/l) + NAA (0.1 mg/l) + CW (10%)

**Micropropagation via somatic embryo culture technique:** Shoots from embryos were used as micropropagation material. Shoot tips and stem nodes were propagated on the medium: WPM + BA (0.1 mg/l) + IBA (0.1 mg/l)

**2.3 Data analysis**

The experiment was arranged in 3 replicates, each replicate had 3 triangular flasks or 5 petri dishes, each triangular flask or each petri dish was inoculated with 5 samples. The collected data were processed using MSTAT-C statistical software.

**3. RESULTS AND DISCUSSION**

**3.1 Callus culture initiation**

Most of the cultures produced somatic cells. The stem, leaf and root cultures on A4, A8 and A12 media all produced high somatic cell production. Vitamin B5 (5 mg/l) and Ca-panthothenate were added (4 mg/l) to the culture medium showed somatic pro-embryonic cell production (Table 1) (Lim Sooi Ping *et. al.,* 2004; Srinivasan *et. al.,* 2012; Akram and Aftab, 2016). Callus milky white color was chosen that was soft as cream having roud-shape instruction (Figure 1) (Figure 1) (Van Minh, 2020)

**Table 1: Study on the ability to create callus cells from stems, leaves and roots**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **A** | BA (mg/l) | NAA (mg/l) | Ki (mg/l) | Biomass (mg/cluster) | Color of cell cluster |
| Callus performance form shoot | | | | | |
| 1 | 1 | 0.5 | 1 | 794 c | Clear white |
| 2 | 1 |  |  | 280 a | Clear white |
| 3 | 1 |  | 1 | 462 b | Clear white |
| 4 | 1 | 0.5 | 1 | **825 d** | Milky white |
| M | | | | 590,2 |  |
| CV (%) | | | | 8,2 |  |
| Callus performance from leaves | | | | | |
| 5 | 1 | 0.5 | 1 | 585 c | Green |
| 6 | 1 |  |  | 128 a | Green |
| 7 | 1 |  | 1 | 261 b | Green |
| 8 | 1 | 0.5 | 1 | **672 d** | Green |
| M | | | | 411,5 |  |
| CV (%) | | | | 10,6 |  |
| Callus performance from roots | | | | | |
| 9 | 1 | 0.5 | 1 | 284 c | Clear white |
| 10 | 1 |  |  | 89 a | Clear white |
| 11 | 1 |  | 1 | 165 b | Clear white |
| 12 | 1 | 0.5 | 1 | **328 d** | Milky white |
| Mean | | | | 216,5 |  |
| CV (%) | | | | 12,8 |  |



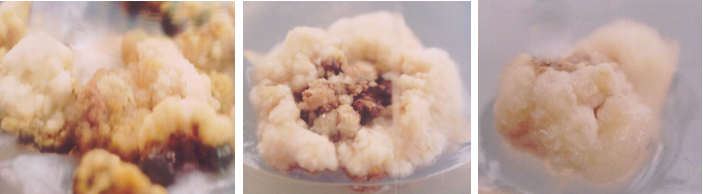
**Figure 1:** Callus formation with shoot tip (left), leaf (middle) and root (left) explants on basic medium MS + BA (1 mg/l) + Kinetin (1 mg/l) + NAA (0.5 mg/l) after 30 days of culture. Callus sourced shoot tip is milky white color.

**3.2 Culturing embryogenic callus cells on agar medium**

With the ability to generate strong callus cells (Table 1), the second stage is the stage of studying the ability to culture differentiated callus cells to form embryogenic callus cells. The research results show that the tightly structured embryogenic callus cells arise on the milky white tissue mass on medium B2 (Table 2). Treatments B1 and B3 give low embryogenic callus cell generation. Callus cells subcultured were milky white color as cream with round instruction cells. But, caullus cells sourced from shoot tip were more bigger round shape structure than the others (Figure 2) (Imran Kozgar and Shahzad, 2012; Akram and Aftab, 2016; Van Minh, 2020). Callus cells sourced from shoot tip were selected for far studies.

**Table 2: Study on the ability to culture embryonic callus cells on agar medium**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **B** | 2.4D  (mg/l) | Biomass starting  (mg/cụm) | Biomass after culture (mg/cụm) | Biomass proliferation  (mg/cụm) | Biomass proliferation  rate | Pro-embryo  (+/-) | Color of cell cluster |
| 1 | 0,5 | 672 a | 958 a | 286 a | 1.43 | - | Clear white |
| 2 | 1,0 | 645 a | 1285 b | 640 b | 1.99 | + | Milky white |
| 3 | 2,0 | 625 a | 1786 c | 1161 c | 2.86 | - | Clear white |
| Mean | | 647,3 | 1343,0 | 695,6 | 2.09 |  |  |
| CV(%) | | 6,5 | 7,2 | 9,8 |  |  |  |



**Figure 2:** Embryogenic callus cells of shoot tip (left), leaf (middle) and root (right) on medium B2: MS + 2.4D medium (1 mg/l) after 30 days of culture. Callus sourced shoot tip is milky white color.

**3.3 Rapid multiplication of embryogenic callus cells on liquid medium**

During the process of culturing embryogenic callus cells, the cells that develop surround the tissue mass, and it is necessary to study to separate the embryogenic callus cells from the tissue mass. The research results show that the fine cell clusters begin to separate after 7 days of culturing. The embryogenic callus clusters completely separate from the tissue mass after 30 days of culturing to form a suspension on the C4 culture medium (Table 3) (Figure 3). The C4 medium is used as a medium for rapid proliferation of embryogenic callus clusters and suspension cells that rapidly increase their biomass after 30 days of culturing with biomass starting of 624 mg/cluster (C5) (Table 3) (Imran Kozgar and Shahzad, 2012; Akram and Aftab, 2016; Tambarussi *et. al.,* 2017; Van Minh, 2020).

**Table 3: Study on the ability to culture, separate and multiply embryogenic callus cells in liquid medium**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Isolated culture of embryogenic callus cells (1) after 30 days | | | | | | |
| **C** | BA  (mg/l) | NAA  (mg/l) | Ki  (mg/l) | 2.4D  (mg/l) | TDZ  (mg/l) | Biomass of embryogenic callus cells (mg/cluster) |
| 1 | 0.5 | 0.2 | 0.5 |  |  | 2568 c |
| 2 | 0.5 |  |  |  |  | 1172 a |
| 3 |  |  |  | 0.3 |  | 2865 d |
| 4 | 0.5 | 0.2 |  |  | 0.2 | **1280 ab** |
| Mean | | | | | | 1971,2 |
| CV(%) | | | | | | 8,7 |
| Rapid propagation of embryogenic callus cells biomass (2) afte 30 days | | | | | | |
| **C** | Biomass initiating  (mg/cluster) | | Biomass after culture  (mg) (2) | Biomass proliferation  (mg) | Biomass proliferation  rate | Color of embryogenic callus cells |
| 5 | 624 | | 9261.5 b | 9199.1 | **14.74** | Light milky white |
| 6 | 1248 | | 4709.8 a | 4585.0 | 3.67 | Light milky white |
| 7 | 1872 | | 13698.2 c | 13511.0 | 7.21 | Light milky white |
| Mean | | | 9223.1 | 9098.3 | 8.54 |  |
| CV(%) | | | 13.6 | 7.8 | 1.03 |  |

(1) After 30 days of culture (2) Media: Basic MS + BA (0.5 mg/l) + TDZ (0.2 mg/l) + NAA (0.2 mg/l)



**Figure 3:** Study on the ability to culture, separate and multiply embryogenic callus cells in liquid medium C4: MS + BA (0.5 mg/l) + TDZ (0.2 mg/l) + NAA (0.2 mg/l) after 30 days cullturing. Clusters and cells suspension were after 7 days (left), 14 days (middle) and 30 days (right)

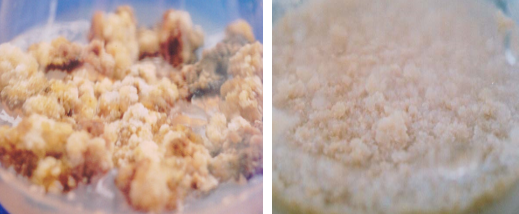
**3.4 Layering pro-embryogenic cells on agar medium**

With the culture sample being clusters of somatic pro-embryogenic cells after the stage of rapid proliferation in liquid medium, cultured in the differentiation medium into somatic embryos. The research results showed that somatic pro-embryonic cells differentiated into somatic embryos after 30 days of culture on agar medium D4. Cell clusters and cell suspensions go into division to form cell clusters on D4 media (Table 4) (Figure 4).

To regenerate teak somatic embryos, after many unsuccessful regeneration studies, it is necessary to study the conditions media for layering somatic embryo cells; especially after the stage of cultivation of the heart shape to differentiate into mature embryos. (Table 4). The research results showed that somatic embryos from white color was changed green color on medium D7 after 30 days, and D5 was till pro-embryo (Table 4). Cultivation of cell clusters on agar and paper bridge show that its more early differentiate to mature embryo on paprer bridge (Figure 5) (Imran Kozgar and Shahzad, 2012; Akram and Aftab, 2016; Tambarussi *et. al.,* 2017; Van Minh, 2020)

Table 4: Study on the ability to culture somatic embryonic cells and the conditions for layering embryogenic cells

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Embryogenic callus cells culture induction media (1) after 30 days | | | | | | | |
| **D** | BA  (mg/l) | NAA  (mg/l) | Ki  (mg/l) | 2.4D  (mg/l) | TDZ  (mg/l) | Pro-embryoge cell | Embryogenic cell |
| 1 | 0.5 | 0.2 | 0.5 |  |  | + | - |
| 2 | 0.5 |  |  |  |  | + | - |
| 3 |  |  |  | 0.3 |  | + | - |
| 4 | 0.5 | 0.2 |  |  | 0.2 | + | + |
| Condition media for Embryogenic cells culture performance (2) after 30 days | | | | | | | |
|  | BA (mg/l) | NAA (mg/l) | TDZ (mg/l) | Globula | Heart | Mature embryo | |
| 5 | 0.5 | 0.2 |  | + | - | - | |
| 6 | 0.5 |  |  | + | - | - | |
| 7 | 0.5 | 0.1 | 0.2 | + | + | + | |



**Figure 4:** Layering pro-embryogenic cells on agar medium D4: MS + BA (0.5 mg/l) + TDZ (0.2 mg/l) + NAA (0.2 mg/l) after 30 days. Cells sourced from cell clusters (left) and cell suspension (right) appear embryogenc cell with round shape and light cream color



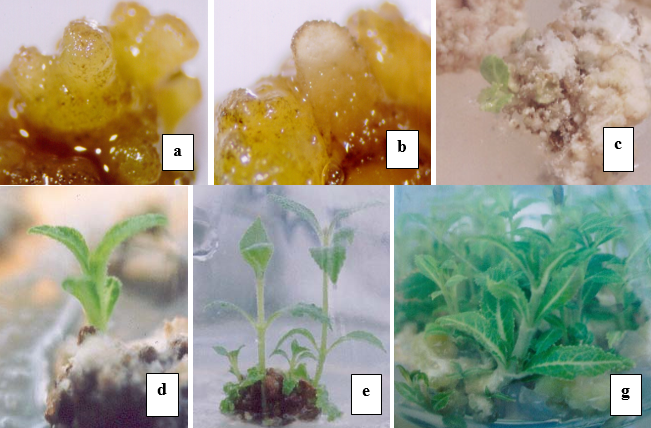
**Figure 5:** Stimulating from somatic embryo cells differentiate into primary mature embryo cell on media D7: MS + BA (0.5 mg/l) + TDZ (0.2 mg/l) + NAA (0.2 mg/l) (left and middle) and green embryo on D5: MS + BA (0.5 mg/l) + NAA (0.2 mg/l) (right) after 30 days. Cultivation of cell cluster on agar (left) and paper bridge (right).

**3.5 Somatic embryo regeneration**

The embryonic stem cell clusters were cultured for regeneration. The results showed that the young shoots regenerated on E7 medium (Table 5) after 120 days of culture. The progree of regeneration from teak embryogenesis in Figure 6: (a) torpedo shape 2weeks (b) cotyledon shape 4ws (c) embryo regeneration 6ws (d) shoots rise 8ws (e) shoot has true leaves 10ws (g) plantlets from embryogenesis 12ws (120 days after culturing) (Baghel *et. al.,* 2008; Imran Kozgar and Shahzad, 2012; Akram and Aftab, 2016; Tambarussi *et. al.,* 2017; Van Minh, 2020)

**Table 5: Somatic embryo regeneration studies on MS medium after 120 days**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **E** | BA  (mg/l) | TDZ  (mg/l) | NAA  (mg/l) | CW  (%) | Regeneration ability (+/-) | Regeneration rate (%) | Shoots  (/cluster) |
| Control |  |  |  |  | - | - | - |
| 1 |  | 0.2 |  |  | - | - | - |
| 2 |  | 0.5 |  |  | - | - | - |
| 3 |  | 0.5 | 0.2 |  | - | - | - |
| 4 |  |  |  | 10 | - | - | - |
| 5 | 1 | 0.5 |  | 10 | - | - | - |
| 6 | 1 | 1.0 |  | 10 | - | - | - |
| 7 | 1 | 1.0 | 0.1 | 10 | + | 85 | 3 |



**Figure 6:** Teak somatic embryo regeneration on media E7: MS + BA (1 mg/l) + TDZ (1 mg/l) + NAA (0.2 mg/l). (a) heart shape embryo 2weeks (b) cotyledon shape embryo 4ws (c) primary shoot regeneration 6ws (d) shoots rise 8ws on MS medium (e) shoot with true leaves 10ws on WPM medium (g) plantlets from embryo 12ws on MS medium (120 days after culturing)

**3.6 Micropropagation via somatic embryo culture technique:**

Multiple shoots from embryos were used as micropropagation materiasl. Shoot tips and stem nodes were propagated on the medium: WPM + BA (0.1 mg/l) + IBA (0.1 mg/l) (Ansari *et. al.,* 2001; Tiwari *et. al.,* 2002; Emilio Mendoza *et. al.,* 2007; Van Minh, 2020)



Figure 7: Teak plantlets from embryo regeneration (left) were multiplication (middle) on media WPM + BA (0.1 mg/l) + IBA (0.1 mg/l) under pilot scale (right)

**CONCLUSION**

Teak is a valuable woody timber tree in industry. It has been restored and micropropagated for reforestation by using plant cell biotechnology. A pilot scale for avoidance of degeneration, regeneration and micropropagation system using somatic embryo technology has been established.

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