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Research Article

The effect of different concentrations of NAA and BAP in promoting *in vitro* regeneration and the acclimatization of the leopard orchid (*Ansellia Africana* lindl)

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ABSTRACT

***Ansellia Africana* lindl is an endemic orchid species in sub-Saharan Africa classified on the red list as "vulnerable" owing to its overexploitation for medicinal purposes? *In vitro* propagation has been a viable alternative for conservation and propagation of endangered orchid species. Many culture media are used in *in vitro* propagation of orchid; however, there is still no specific formulation for *Ansellia Africana* lindl. The aim of this work was to evaluate the effect of different treatment of the growth regulators Naphthalene Acetic Acid (NAA) and Benzylaminopurine (BAP) on the number of leaves, number of shoots, shoot length, number of roots and root length in the *in vitro* cultivation of *Ansellia Africana* lindl. The BAP and NAA treatment caused differentiated responses in *Ansellia Africana* lindl. *In vitro* regeneration. Treatment with BAP 1.0 mg/L and NAA 2.0 mg/L showed the best results in most of the parameters analyzed in *in vitro* development of plantlets of the orchid. This treatment could be used in micro propagation of this orchid and contribute to its conservation. In acclimatization under *ex vitro* conditions, the substrate with *Sclerocarya birrea* trunk bark+sand provided the highest percentage of plantlets survival (66.6%) followed by rice husk+sawdust+coco mix with 61.90% and by Afzelia quanzensis trunk bark+coconut fiber when compared to control.**

Keywords: Auxin, conservation, Cytokinin, Endemic, Micropropagation, Acclimatization, Orchidaceae

INTRODUCTION

Ansellia Africana lindl, known as "leopard orchid" is a species endemic to Africa. It is found in Nigeria, Tanzania, Angola, Mozambique and South Africa [1]. It is at risk of extinction and it is classified in the red list of plants as “vulnerable” by the international union for the conservation of nature because of overexploitation for ornamental and medicinal purposes, and the reduction of their natural habitats [2].

Traditionally, *Anselia africana* is used as an antidote to fight bad dreams by the Zulus of South Africa, while in Zambia, infusions prepared from the stem and leaves are used to treat madness [3].

The similarity of other species of orchids to their reproduction through seeds is quite limited because of the thousands of seeds produced in a capsule, only about 1 to 5% germinate owing to absence of endosperm the vegetative growth of seedlings originated by this means is very slow [4]. Macro propagation of orchids is carried out by dividing rhizomes, which represents an easy and safe way from a genetic point of view; however, it is not commercially viable because it has a low reproductive capacity and requires a period of between two and eight years

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to obtain an adult individual [5]. On the other hand, micro propagation, that is, *in vitro* cultivation, has been shown to be an effective alternative in the multiplication of orchids and the restoration of the natural population [6]. For the specie *Ansellia Africana* some *in vitro* protocols were established but with seeds in a study carried out by Vasudevan and Staden in which was evaluated the *in vitro* symbiotic seed germination and seedling growth.

This result suggests that the germination was affected more by the growth medium used for culturing the seeds rather than the sodium hypochlorite concentrations. The use of P668 medium is recommended for achieving the best germination in mature seeds of *A. africana*. Auxins and cytokinins are the classes of growth regulators most frequently used in *in vitro* culture. Their concentration and combination in the culture medium is an important factor that determines the successful regeneration of plants [7]. The cytokinin 6-Benzylaminopurine (BAP) and auxin α-Naphthalene Acetic Acid (NAA), were tested with good results for the *in vitro* propagation of several species of orchids, such as *Dendrobium sp., Dayaoshania cotinifolia, Epidendrum secundum* and *Otochilus albus* lindl [8]. However, each genotype presents a different answer to this balance established between growth regulators [9]. Although different studies were performed with the objective to stabilize protocols of *in vitro* regeneration and acclimatization of *A. africana* there is still a need to stabilize low cost and effective protocols for *in vitro* regeneration and acclimatization. Those would be helpful to contribute to reduce the pressure over natural habitats of *A. Africana* and also to the conservation of this specie under risk of extinction.

Acclimatization, a process through which plantlets produced under controlled conditions are transferred to a transitional environment, before being taken to the field, is one of the essential points that guarantee success in obtaining seedlings from micro propagation [10].

At this stage, the quality of the plantlets in production depends on factors such as humidity, temperature, light and a substrate that guarantees better adaptation. These substrates significantly influence the architecture of the root system and the nutritional status of the plant [11]. In a study with *A. africana* seedlings developed on P668 medium showed a better growth response in terms of leaf growth, root growth and fresh and dry weights per plant after 12 weeks of *ex vitro* growth in a mist house with 90% relative humidity [12].

The present work aims attempt to improve *in vitro* regeneration and acclimatization of *Ansellia Africana* lindl.

- Leopard orchid is a red list vulnerable endemic orchid in the African continent.
- Orchid's physiological parameters exhibited high response to growth regulators.
- Specific formulation culture media for *Ansellia africana* lindl. *In vitro* cultivation.
- Growth regulators cause differentiated responses in orchid *in vitro* regeneration.
- *In vitro* propagation can contribute to conservation of the leopard orchid.

MATERIALS AND METHODS

Regeneration *in vitro*

Seeds of *Ansellia Africana* lindl. Were collected at the Mozambique agricultural research institute (IIAM) botanical garden. Those seeds were submitted to asepsis using 1.5% of active sodium hypochlorite solution for 10 minutes, and then three consecutive washes were performed with distilled water.

The seeds of *A. africana* were Africana was placed in the medium for germination, and growth of plantlets to obtain explants (Figure 1). The culture medium Murashige and Skoog (MS) was supplemented with 100 mg L^{-1} of inositol, 3% sucrose and 0.5% agar. The pH of the media was adjusted to 5.7 before adding the agar and before sterilization at 120°C and 1 atm for 20 minutes.

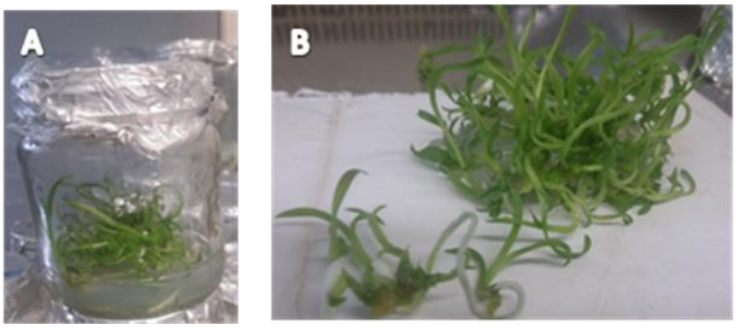

Figure 1. Plantlets of *Ansellia Africana* lindl. Used for inoculation. (A). In the culture environment and; (B). Matrixes used to obtain the explants.

The medium most often used in the *in vitro* cultivation of orchids is the MS culture medium for regeneration, was prepared at a concentration of 4.48 g/L, with 10 g/L of bacteriological agar (Himedia, India) supplemented with 30 g/L sucrose and pH adjusted to 5.7. The media were transferred to sterilized (at 132ºC for 15 minutes) transparent glass containers (Height=5 cm, mouth diameter=5 cm with a capacity of 50 mL, containing 25 mL of culture medium [13].

These containers were sealed with aluminum foil and cling film and then autoclaved at 121°C and 1 atm pressure for 15 minutes. Seven treatments (control, and six combinations of the growth regulators BAP and ANA) were established (Table 1).

Table 1. MS culture medium supplemented with different concentrations of BAP and NAA.

Treatments	BAP (mg/L)	NAA (mg/L)
Control	**0**	**0**
1	1	1
2	1.5	1
3	1	1.5
4	1.5	1.5
5	1	2
6	1.5	2

The seedlings of ½ cm in height were inoculated in the media stored in a growing room at 28°C with a photoperiod of 16 hours and light intensity of 20.0 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density from two fluorescent white lamps of 20 W each, approximately 30 cm distant from the culture glass, for 30 days. After 30 days, the plantlets were removed from the glass and evaluated under aseptic conditions the leaves, shoots and roots were counted, and root length and stem elongation measured using a graduated ruler. The experimental design was completely randomized, and the treatments were seven with 20 repetitions for each.

The statistical analysis was performed using the SPSS software program, version 20. The data were analyzed by Analysis of Variance (ANOVA one way). The comparison between the means of the treatments was made by the Tukey test ($p<0.05$).

Acclimatization

Under *ex vitro* conditions, the substrates were moistened and mixed in a ratio of 1:1:1 and arranged according the Table 2. For each treatment were used 21 plantlets.

Table 2. Substracts composition of different treatments used for acclimatization of *Ansellia Africana* plantlets obtained through micro propagation.

Treatment	Composition
Control	Coco-mix+coarse sand
T1	*Sclerocarya birrea* trunk bark+sand
T2	*Sclerocarya birrea* trunk bark+sand+coconut fiber
T3	*Afzelia quanzensis* trunk bark+coconut fiber
T4	Rice husk+sawdust+coco mix

The substrates were placed in 200 ml transparent plastic disposable cups and on which orchid plantlet were transferred, preceded by washing the roots with water to remove adhering agar. To ensure the conservation of relative humidity, the recipients were covered with transparent plastic (humidity chamber) of 200 ml volume and the gradual reduction of humidity was carried out until the 15^{th} day by opening holes in the humidity chamber. At the end of 120 days, the plantlets were evaluated in terms of percentage of survival.

Data were organized in Microsoft Excel 2013 software and graphs were elaborated using the graph pad prism version 7 software.

RESULTS

Figure 2 illustrates the effect of the combination of BAP and NAA growth regulators on leaf root and shoots numbers in plantlets of *Ansellia Africana* lindl.

The number of leaves was statistically significative in all treatments ($p<0.05$). The treatment with a concentration of 1 mg/l of BAP+2 mg/l of NAA caused an increase of 38.8% in leaf number compared to the control. The highest reduction in the number of leaves (33.6%) was observed in the combination of 1.5 mg/l of BAP+1 mg/l of NAA when compared to the control (Figure 2).

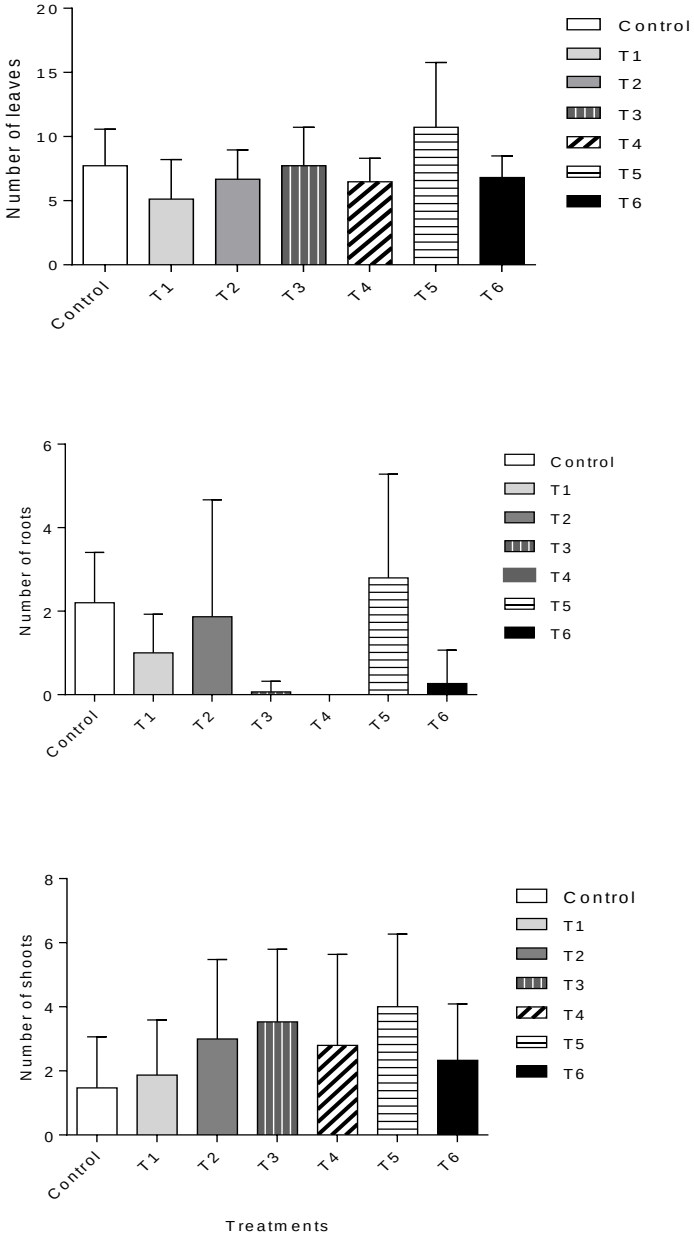

Figure 2. (A). Effect of growth regulators (BAP and NAA) on the development of leaf; (B). Root and; (C). Shoot numbers in plantlets of *Ansellia Africana* lindl. Control (0 mg/l BAP+0 mg/l NAA), T1 (1 mg/l BAP+1 mg/l NAA), T2 (1.5 mg/l BAP+1 mg/l NAA), T3 (1 mg/l BAP+1.5 mg/l NAA), T4 (1.5 mg/l BAP+1.5 mg/l NAA), T5 (1 mg/l BAP+2 mg/l NAA) and T6 (1.5 mg/l BAP+2 mg/l NAA). The bars represent the mean of 20 individual plantlets ± standard deviation.

The number of roots was statistically different in all treatments ($p<0.05$). The treatment with a concentration of 1 mg/l BAP+2 mg/l NAA caused an increase of 27.3% in root number compared to the control. In the treatment using 1:1.5 mg/L of BAP and NAA no regeneration of roots was observed.

The effect of the treatment BAP and NAA growth regulators in the number of shoots of *Ansellia Africana* lindl.

A significant increase in the number of shoots in all treatments was found ($p <0.05$); the highest increase (174%) was observed in the treatment 1 mg/l BAP + 2 mg/l NAA.

Figure 3 shows the effect of the combination of BAP and NAA growth regulators on root length in plantlets of *Ansellia Africana* lindl. A significant reduction of the roots length in all treatments was found ($p<0.05$); the lowest decrease (61.6%) was observed in the treatments of 1:2 mg/l of BAP and NAA.

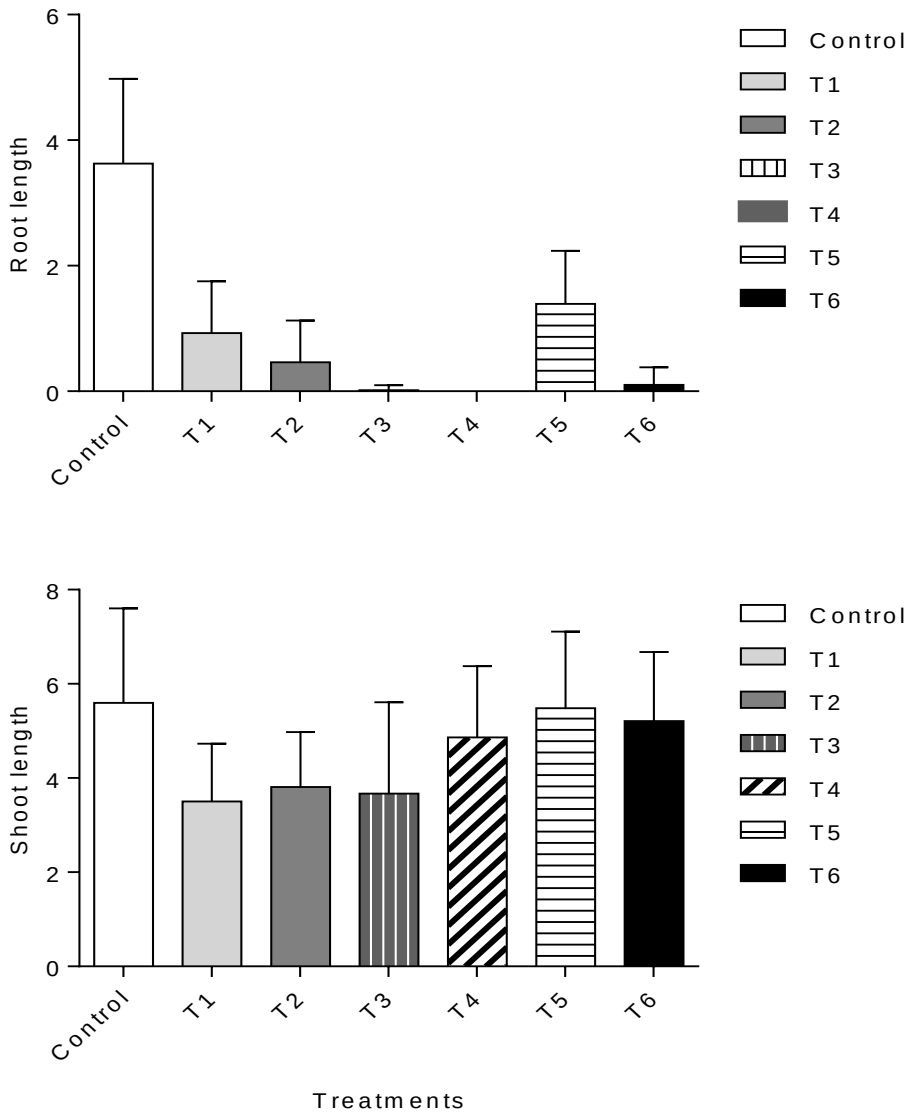

Figure 3. (A). Effect of growth regulators (BAP and NAA) on root length and; (B). Stem elongation in plantlets of *Ansellia Africana* lindl. Control (0 mg/l BAP+0 mg/l NAA), T1 (1 mg/l BAP+1 mg/l NAA), T2 (1.5 mg/l BAP+1 mg/l NAA), T3 (1 mg/l BAP+1.5 mg/l NAA), T4 (1.5 mg/l BAP+1.5 mg/l NAA), T5 (1 mg/l BAP+2 mg/l NAA) and T6 (1.5 mg/l BAP+2 mg/l NAA). The bars represent the mean of 20 individual plantlets ± standard deviation.

The control reduced the shoot length; it was statistically different in all treatments ($p<0.05$) and showed the lowest decrease (3.6%) in the treatment 1:2 mg/l of BAP and NAA.

In acclimatization, T1 provided the highest percentage of plantlet survival (66.6%) followed by T4 with 61.90%, T3 with 52.38%, T2 with 42.84% and control with 33.3% [14-20].

DISCUSSION

The different treatments of BAP and NAA led to different leaf root and shoot growth responses in the orchid *Ansellia Africana* lindl [21,22]. Similar results were found by studying *Cattleya xanthine* (lindl.) Van den Berg, an endangered Brazilian orchid, under BAP and NAA treatments.

We found that the treatment with a concentration 1 mg/l BAP+2 mg/l NAA increased the leaf number in 38.8% compared to the control. This is in line with the results of Mengarda, et al. in a study with *Brassavola tuberculata* Hook, an endemic Brasilian orchid; they observed an increase of 33.3% in different NAA and BAP combinations. It was observed that all the combinations with a low concentration of NAA and high concentration of BAP induced an increase in leaf number [23,24]. These results may be due to the physiological effect of the cytokinin in promoting cell division and differentiation and are also in accordance with many other studies using different orchid species (Figure 4) [25].

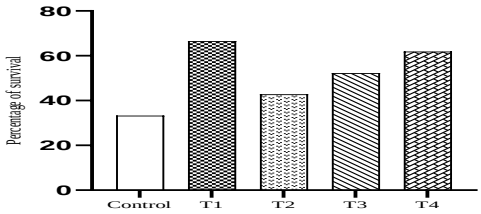

Figure 4. Effect of different substrates on the percentage of survival in seedlings of *Ansellia Africana* lindl. Control treatment coco mix+ coarse sand, T1 (*Sclerocarya birrea* trunk bark+sand), T2 (*Sclerocarya birrea* trunk bark+sand+coconut fiber), T3 (*Afzelia quanzensis* trunk bark+coconut fiber) e T4 (rice husk+sawdust+coco mix) each bar represents the mean of 21 plantlets.

The treatment with a proportion of 1 mg/l BAP+2 mg/l NAA showed an increase of 27.3% in root number compared to the control. Goswami, et al. found an increase of 267% in root number using the combination 2.5 mg/l BAP+0.5 mg/l NAA. Furthermore, in several orchid species, an increase in the concentration of auxins in the culture medium promoted the formation of roots [26-28]. This increase is due to the key role that auxins play in cell signaling, leading to plant growth and development [29,30].

In the treatment 1 mg/l BAP+1.5 mg/l NAA, root regeneration was observed. This result may be explained by the excessive concentration of auxin in the growth medium compromising the rhizogenesis [31]. Recently Fendrych proved that auxins inhibit root growth *via* a non-transcriptional branch of the canonical auxin receptor in a Transport Inhibitor Response 1 (TIR1).

A significant reduction of the root length in all treatments was found; the lowest decrease (61.6%) was observed in the treatment 1 mg/l BAP+2 mg/l NAA. This is in line with Juras who found a reduction about 30% in *Cattleya xanthine* (lindl.) To the contrary, higher concentrations inhibited root elongation in several plant species [32]. This result can be attributed to the possibility that the natural levels of auxin and cytokinin in the plants were sufficient to stimulate the development of root length. According to Olatunji, the need to supply growth regulators to the culture media is related to the endogenous quantity of each species and each seedling and is also dependent on the environment, the transport efficiency of the regulator and the metabolism. All this suggests the need of an adequate balance of BAP and NAA, as those have opposite effects on the rhizogenesis; a higher concentration of auxins promotes the formation of roots and a higher concentration of cytokinins inhibits it [33]. The optimal proportion of auxins and cytokinin depends on the plant species used for micro propagation [34].

The best shoot length was observed in the absence of the plant growth regulators (control treatment), which may be explained by the presence of natural endogenous hormones. The lowest decrease (3.6%) was observed in the treatment 1 mg/l BAP+2 mg/l NAA. Tikendra observed a similar response in shoot development in the treatment BAP and NAA in the orchid *Dendrobium thyrsiflorum* Rchb.f. This is contrary to the results found by Bhowmik and Rahman, with an increase of 175% and 203% in the treatments 1 mg/l NAA+1.0 mg/l BAP and 0.5 mg/l NAA+1.0 mg/l BAP, respectively in shoot length of *Dendrobium palpebrae* lindl. Our results may be due to the fact that the induction or inhibition of *in vitro* morphogenetic processes depends on the balance and interaction between endogenous and exogenous growth substances.

The highest increase in the number of shoots (174%) was found in the treatment 1 mg/l BAP+2 mg/l NAA. The same pattern was found by Balilashaki using the treatment of 2 mg/l BAP and 0.5 mg/l, which was the most appropriate medium for rapid micropropagation of a large number of vegetative shoots. The increase in shoot number was almost 17 times higher in the treatment of 0.5 mg/l NAA+0.5 mg/l BAP. In a study with *Cymbidium aloifolium* (L). Sw., 1 mg/l BAP+1.0 mg/l NAA treatment was found to be optimum for the highest shoot formation. These results are probably related to a higher BAP concentration leading to adventitious shoot formation, auxiliary shoot proliferation through the stimulating cell elongation or division and growth of lateral bud and also cell cycle control. However, Tikendra ,et al. observed an inhibitory effect on shoot number in *Dendrobium thyrsiflorum* Rchb.f. in the treatment of 1 mg/l of BAP+1 mg/l of NAA, once again demonstrating the different species' response to growth regulators.

Also high percentages of survival were reported by Ichinose, for species of *Sphronitis cernua* lindl and *Brassavola fragellaris* acclimatized and cultivated in a substrate composed of a mixture of coconut fiber and sphagnum, the survival percentages were between 60 and 80% for 120 days.

When analyzing the relationship between the chemical characteristics of the substrates and the percentage of plantlet survival, in T1 high levels of calcium and magnesium are observed, which were higher in relation to the coco mix substrate and *Afzelia quanzensis* trunk bark, and it was verified that there were also high levels of Nitrogen in the treatment that were superior to coco mix.

CONCLUSION

The BAP and NAA treatments caused differentiated responses in *Ansellia Africana* lindl. In *in vitro* regeneration.

The treatment 1 mg/L BA+2 mg/L NAA was the best for the regeneration of *Ansellia Africana* lindl. In most of the physiological parameters evaluated.

The combination of *Sclerocarya birrea* trunk bark+sand was the most suitable substrate for the vegetative development of *Anselia Africana* lindl. In the acclimatization phase.

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