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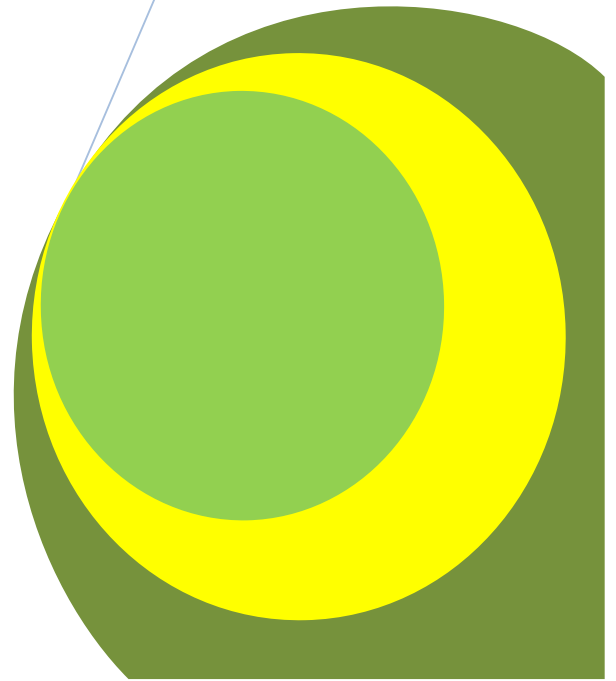
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Introduction of the neMDH gene in *Urochloa brizantha* for Aluminium Tolerance in Agronomic Assays

Flavio Rocha¹, Keila Maria Roncato Duarte^{*2},
Luiz Humberto Gomes¹, Waldssimiller Teixeira Mattos³,
Simone Possedente Lira¹, Paulo Bardauil Alcantara³

¹Laboratório de Química Orgânica de Produtos Naturais, Departamento de Ciências Exatas, Escola Superior de Agricultura "Luiz de Queiroz", USP, Piracicaba, 13418-900, São Paulo, Brazil.

²CPD Genetic and Animal Reproduction, Institute of Animal Science and Pastures, APTA/SAA Nova Odessa, 13460-000, São Paulo, Brazil.

³CPD Pastures and Nutrition, Institute of Animal Science and Pastures, APTA/SAA Nova Odessa, 13460-000, São Paulo, Brazil.

*Corresponding Author's Email: keiladuarte@globo.com

ABSTRACT

Agricultural production in acid soils constituting about half of the total arable land on Earth, is limited by aluminium toxicity, which is manifested by root growth reduction, decreasing nutrients and water absorption by the plant. This work evaluated aluminium resistance in genotypes of *Brachiaria brizantha* cv. MG4, transformed with the neMDH gene, by using a nutrient solution for agronomic evaluation and gene integration confirmation by PCR. Three from the five confirmed transformed genotypes of *B. brizantha* cv. MG4 with the neMDH gene presented tolerance to aluminium solutions (111 $\mu\text{mol L}^{-1}$ and 444 $\mu\text{mol L}^{-1}$). Two from those three genotypes also showed higher dry weight production in comparison to the wild-type genotype.

Keywords: *Brachiaria*, Tolerance, Aluminum, Soil, Transgenic, neMDH.

INTRODUCTION

In acid soils, which represent 50 % of arable land on Earth (PANDA et al., 2009), soluble aluminium mainly as Al^{+3} which is phytotoxic to the plasmatic membranes and damages the root development. Due to the decrease in water and nutrient absorption, plant development does not occur as usual in acid soils, compromising further events of plant growth (Kochian, 1995; Kochian et al., 2004).

Studies have shown that the most common and natural mechanism to avoid Al toxicity is through organic acid exudation from the roots as malate, oxalate or citrate. These organic anions are responsible for the chelated Al form in the rhizosphere, preventing penetration into the roots (Ryan et al., 1995).

Aluminium tolerance is rarely a selected character on plant breeding programmes, thus the intrinsic tolerance is a random event or sub product from the selection of other characters (McNeilly, 1994). The breeding efficiency for aluminium resistance depends on the gene bank variability for the plant species, which can be very short for some crops and pastures (Delhaize et al., 2004).

Exogenous gene introduction or endogenous genes over-expression guarantee a significant increase in tolerance to high concentrations of soluble aluminium in soils for several crops (De La Fuente et al., 1997; Tesfaye et al., 2001; Anoop et al., 2003; Delhaize et al., 2004).

Miller et al. (1998) cloned the complete coding sequence (Cds) from DNA 1645 bp of the neMDH gene (nodule-enhanced malate dehydrogenase precursor, access AF020273.1) from alfalfa (*Medicago sativa*). This enzyme intensifies citrate, oxalate, malate, succinate and acetate secretion in roots, promoting Al chelating in the rhizosphere and increasing Al tolerance (Teschfaye et al., 2001).

Forage crops, consisting of around 80 % *Brachiaria* grasses, are of great importance for the tropical Americas, Oceania and part of the Asiatic countries, being the base for beef and milk production, as well as for sheep and equine rearing (Macedo, 2006).

This study aimed to verify the Al tolerance incorporated by the genetic transformation of palisade grass (*Brachiaria brizantha*, MG4 cultivar) through the introduction of a stable neMDH gene, using the *Agrobacterium tumefaciens* technique associated with micro projectile bombardment (biolistic method), thus evaluating the

molecular characterisation of the transformed products and progenies in relation to toxic Al levels in a nutritional solution.

MATERIAL AND METHODS

Plant transformation

Palisade grass seeds (*Brachiaria brizantha*, MG4 cultivar) were sterilised and immersed in water until germination. *B. brizantha*, with co-cultivation of scutellum with *A. tumefaciens* EHA101 pLG121-TaCIC (*ZmUbi pro*) and biolistics of zygotic embryos. The vector inserted was pCambia1301 with the neMDH gene (nodule-enhanced malate dehydrogenase precursor) for Al tolerance - access AF020273.1, under *Zea mays* ubiquitin promoter (*Ubi1*) allowing expression on Monocotyledoneae and giving hygromycin resistance (HPT) and GUS reporter gene. Transgenic embryos were screened by antibiotic hygromycin resistance ($100 \mu\text{g mL}^{-1}$) (Quecini et al., 2008).

PCR Molecular Analysis

For the molecular analysis, genomic DNA was extracted from leaves (100 mg) using the CTAB method (Doyle and Doyle, 1987) in a polymerase chain reaction; forward and reverse primers used were specific to the neMDH gene ORF's, 5'- TGGATGTTCCAGTTGTTGGA -3' and 5'- GTGCACGAAGAGACGATTCA -3', respectively. Amplification size was 222 pb for the neMDH. Reaction protocol was performed using 1x Taq Buffer (Fermentas), 3.0 mM MgCl_2 , 0.2 mM from 4dNTP mix, 100 ng from DNA, 1 U of Taq polymerase (Fermentas), 0.5 μM from each gene specific primers and ultra pure water to the final volume of 25 μL . Initial denaturation (5 min at 94°C) was followed by 30 amplification cycles (30 sec at 94°C , 60 sec at 55°C and 60 sec at 72°C) and a final extension of 5 min at 72°C . Negative controls were performed with genomic DNA of non-transformed plants. Analysis of PCR products were visualised in 1 % agarose gel 1% (w/v) after ethidium bromide staining. DNA standard was a 1kb GeneRuler™ DNA Ladder (Fermentas).

Progenies Analysis for Al tolerance and Phenotypic Evaluation

For the Al tolerance test, a randomised block design was used in a factorial scheme 6×3 , six genotypes (five transformed plants and one wild) and three Al concentrations ($0 \mu\text{M L}^{-1}$, $111 \mu\text{M L}^{-1}$ and $444 \mu\text{M L}^{-1}$), with two blocks and five replications.

The agronomic Al tolerance test was carried out in a 30 L nutritional solution, pH 4.5, using PVC boxes. Seeds obtained from T0 palisade grass plants (Figure 1a) were scarified using sulphuric acid, followed by four washing steps in water. They were later sown in plastic cartridges containing washed sand, and irrigated twice a day (Figure 1b). After one week, the sand was replaced by artificial foam and the seedlings were splitted into three treatments with Al dissolved: (A) $0 \mu\text{M L}^{-1}$; (B) $111 \mu\text{M L}^{-1}$; (C) $444 \mu\text{M L}^{-1}$, constantly homogenised by aerators (Figure 1c). The nutrient solution was prepared as described by Furlani and Furlani (1988).

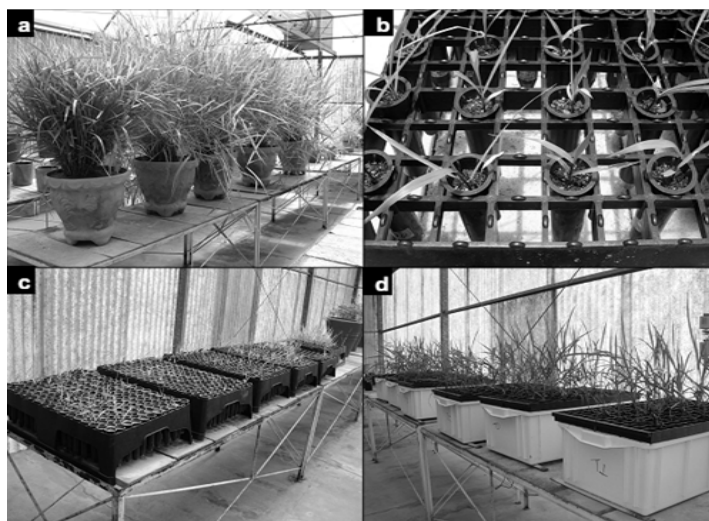


Figure 1: Agronomic evaluation for Al tolerance: seeds obtained from *Brachiaria* transformed genotypes F0 (a); seeded in plastic tubes with sand (b, c); Seedlings removed from sand, involved in vermiculite and tested into three soluble AL concentrations (d).

After 30 days, replicates of each treatment, within block, were mixed, aerial and root parts were spliced and oven-dried at 65°C for 48 h up to constant weight.

Statistical analysis

Dry weight production from aerial parts and roots were used to determine the variance analysis and the Tukey test at 5% of probability.

RESULTS

Plant Transformation

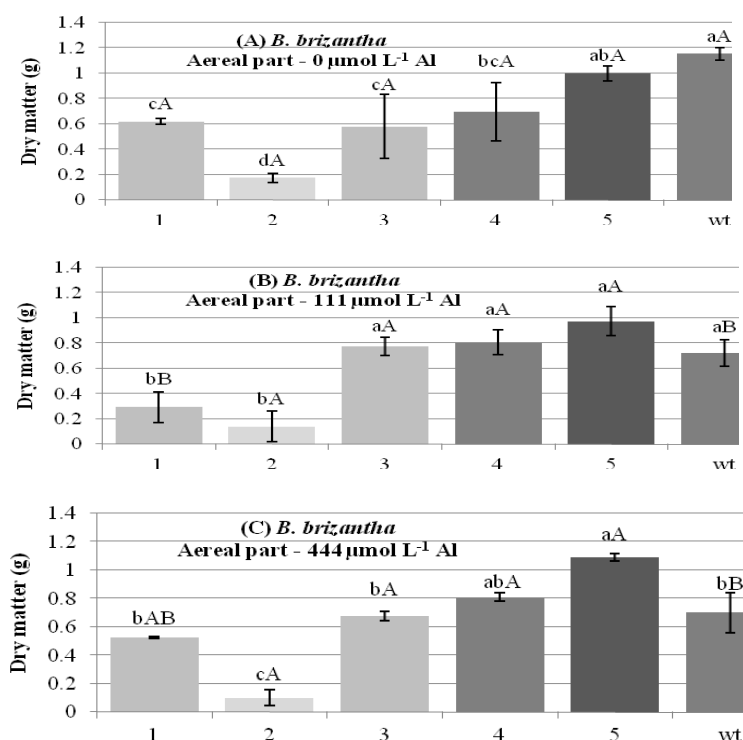
Transformation efficiency was 0.82% (Quecini et al., 2008). Five plants from *Brachiaria* were regenerated in a selective culture media with hygromycin and by the GUS histochemical test.

Molecular analysis

PCR protocol needed to be modified using 6 mM of $MgCl_2$, generating sharp bands, with less specificity. Primer amplification of the neMDH gene, on positive control (plasmid used as transformation vector) regenerated, as expected, a 222 bp band. Therefore, same reaction occurred in the transformed genotypes, showing a 450 bp band, with fade intensity on negative control (non-transformed plant).

Progenies and phenotypic evaluation for Al tolerance

The variation coefficient was 17.00% for aerial dry weight ($P < 0.05$; Figure 2) and 12.03% for root dry weight ($P < 0.01$; Figure 3).



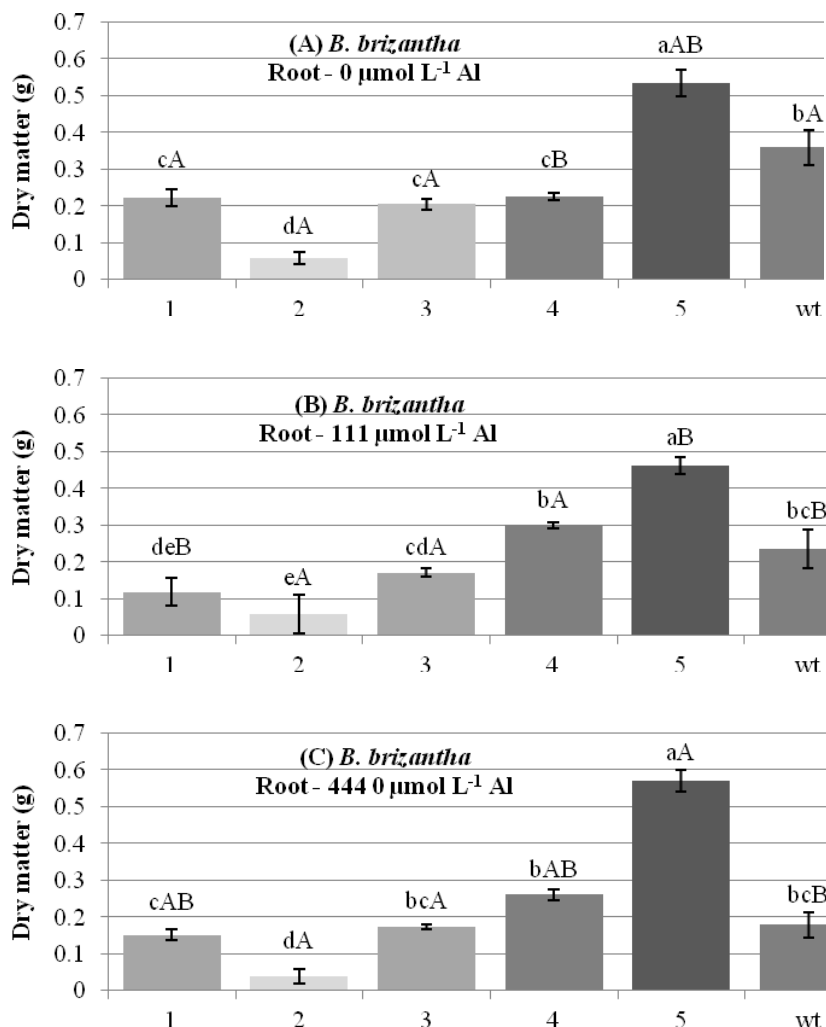
*averages with same low letter – among genotypes and, capital letters for treatment, did not differ statistically by Tukey test at 5%;

Figure 2: Average of aerial dry matter from transformed *B. brizantha* genotypes (1, 2, 3, 4, 5) and wild type (wt), in three different Al concentration solutions.

A negative correlation was observed between the Al increase and dry matter production on aerial part for the genotype 1 (-0.01); 2 (-0.97) and wt (-0.73) and from root for the genotypes 1 (-0.43); 2 (-0.97), 3 (-0.63) and wt (-0.88). Therefore a positive correlation was observed on genotype 3 (0.23); 4 (0.69), and 5 (0.90) for aerial part and genotype 4 (0.23) and 5 (0.58) for roots, using ANOVA.

For the wild-type, on $444 \mu\text{M L}^{-1}$ Al solution, a dry weight reduction of up to 39% for aerial parts and 50% for root weight was observed.

In the solutions with no Al ($0 \mu\text{M L}^{-1}$), the highest dry weight was obtained for genotypes 5 and 6 (Figure 2A), but for root dry weight, genotype 5 showed an increase in the aerial dry weight in comparison to the wild-type (49 % superior) (Figure 3A).



**averages with same low letter – among genotypes and, capital letters for treatment, did not differ statistically by Tukey test at 5%;

Figure 3: Root dry matter average production from *B. brizantha* transformed genotypes (1, 2, 3, 4, 5) and wild type (wt), at three Aluminum concentrations.

In the solution with $111 \mu\text{M L}^{-1}$ Al, higher dry weight production was obtained equally for genotypes 3, 4, 5 and 6, with no differences in comparison to the wild-type (Figure 2B). While the root dry weight was superior for genotype 5 (63 % higher than the wild-type) followed by genotypes 4 and 6, which did not differ statistically (Figure 3B).

For Al concentrations of $444 \mu\text{M L}^{-1}$, the aerial dry weight was superior for the genotypes 5 and 4, in comparison to the wild-type, 34 % and 9 % respectively (Figure 2C). Considering root dry weight, genotype 5 was 109 % superior to the wild-type and genotypes 3 and 4 did not differ from the wild-type ($P > 0.05$), as seeing in Figure 3C.

Higher dry weight production on genotype 1 was $444 \mu\text{M L}^{-1}$ Al, the same as the wild-type genotype for aerial and root parts (Figures 2C and 3C). Genotype 2 was the only one to show a decrease in dry weight production, inferior to the wild-type at all Al concentrations.

Statistically, there were no significant variations (Tukey test, at 5%) for aerial dry weight in genotypes 3, 4 and 5 (Figure 2) and for root dry weight production in genotypes 4 and 5 (Figure 3 and Figure 4), at all Al concentrations, which indicates tolerance at high Al concentrations.

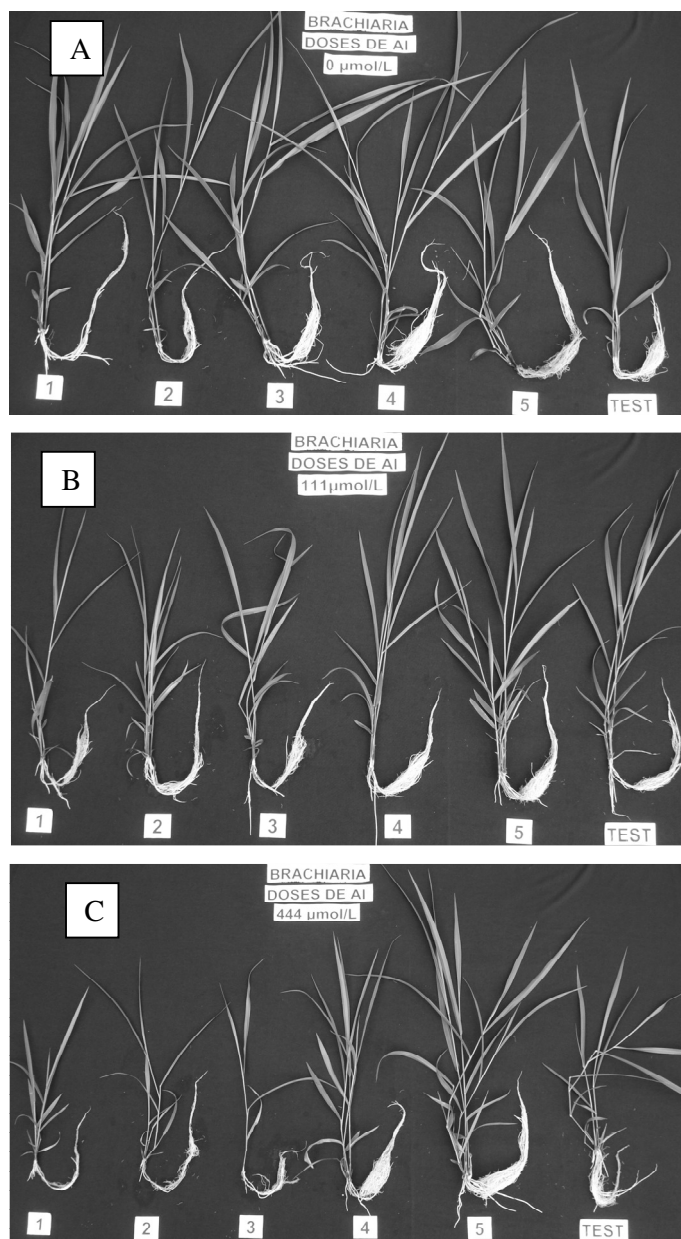


Figure 4: Aerial and root visualization of the five *B. brizantha* transformed genotypes (1, 2, 3, 4, 5) and wild type (TEST), after 30 days in nutrient solution wadded with aluminum, A = $0 \mu\text{mol L}^{-1}$, B = $111 \mu\text{mol L}^{-1}$, and C = $444 \mu\text{mol L}^{-1}$.

DISCUSSION

The difference observed in the size of amplification products, in comparison to the wild-type, can be explained by recombination events, with unknown DNA insertion during transgene integration in *Brachiaria* genotypes.

Petry (2009) found similar results when trying to find transformed soybean genotypes and wild-types using the chitinase gene *chit1* from *Metarhizium anisopliae*. The gene amplification in the wild-type comes from endogenous chitinase from soybean.

According to Minárik et al. (2002) and Miller et al. (1998) the malate dehydrogenase (MDH) enzyme is important to several metabolic pathways, due to its function to catalysis the reversible reduction of oxaloacetate to malate. This enzyme is present in superior plants in several forms of isozymes, with different specificity and cellular location, including the chloroplast, the mitochondria and the peroxisome. The same isozyme form is also present in different species, due to its common origin.

Kohli et al. (1999) explained that after insertion of the transgene in the nucleus, complete integration depends upon proteins from vegetal cells evolved in the DNA replication, its repair system and recombination events. Therefore, exogenous DNA integration is still poorly understood and the position and locus structure can vary among transformed individuals.

Jackson et al. (2001) observed three patterns for transgene integration by biolistic methodology: type I – integration of wide segment, repeated in tandem (identical sequences); type II – great sequences repeated, spaced by DNA sequence and, type III – small sequences repeated in tandem, possibly spaced by unknown DNA. *Urochloa* plants are apomictic, therefore segregation is not expected in T1 generation.

Dissolved aluminium (Al^{3+}) can be found at 10 to 40 $\mu\text{M L}^{-1}$ in acid soils (pH~4.5). These levels are lower when compared to the assay carried out in this study. At nutrient solutions normally used by plant nutritionists, Al micro-molar concentrations are responsible for initial root growth inhibition in about 60 minutes (De La Fuente et al., 1997).

The development of the wild-type decreased in the presence of Al, showing a negative correlation between Al concentrations and plant development, thus corroborating previous studies (Silva et al., 2007; Mazzocato et al., 2002; Portaluppi et al., 2010; Wenzl et al., 2003). Tesfaye et al. (2001) found lower dry weight content in transformed plants using the same gene for transformation, in comparison to the wild-type plant, justifying the increase in citrate and malate exudation by roots, process that demands high energy for the plant cells.

Tolerance to high aluminium concentrations in aerial parts of genotypes 3, 4 and 5 and root parts for genotypes 4 and 5 can be found in data published by Tesfaye et al. (2001), who verified root elongation and biomass increase on transformed genotypes in the presence of aluminium.

Lower Al tolerance, observed in transformed genotype 2, is probably due to multiple transgene insertion, resulting on transgene silencing and therefore, phenotype suppression. How transgene integrates physically in plant genome is a mechanism still poorly understood. The two major methods for plant transformation (*Agrobacterium* and biolistic) generate plants with multiple and rearranged transgene copies at one only physical locus (Lessard et al., 2002). According to Matzke et al. (2000), the transgene expression is inverse from gene copy number, so a high number of copies lead to gene silencing mechanisms, contradicting the usually expected result.

A silencing event for the MDH gene resulting on less biomass agrees with the results obtained by Tomaz et al. (2010), after knockout the mMDH gene from *Arabidopsis thaliana* had plants with noted decrease on growth.

CONCLUSIONS

Transformed genotypes of *Brachiaria brizantha*-neMDH gene presented transgene integration. PCR amplification showed a possible unknown DNA sequence integration in all transformed genotypes, probably by the endogenous MDH gene influence.

Transformed genotypes 3, 4 and 5 were Al tolerant at all Al concentrations, although genotypes 4 and 5 presented higher aerial dry weight production in comparison to the wild-type.

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