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Original Article

Evaluating Morphological, Physiological and Biochemical Responses of Three Amphiploid Brassica Species to Salinity Stress

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Abstract

Brassica occupy third place among the various oilseed species but their productivity, growth and oil production are greatly reduced due to salinity. Amphidiploid Brassica species includes *B. napus* (AC genome), *B. juncea* (AB genome) and *B. carinata* (BC genome) are more tolerant to salinity as they are derived from diploid species which include *B. campestris* (AA, n=10), *B. nigra* (BB, n=8) and *B. oleracea* (CC, n=9). Screening of available local cultivars may facilitate us in identification of varieties suitable for that particular area. Therefore, in the present study different parameters such as growth, electrolyte leakage, K^+/Na^+ ratio, chlorophyll, protein, malondialdehyde and proline content were used to study the effects of 200 mM NaCl for 24 h on the seedlings of available local three amphiploid *Brassica* species i.e., *B. juncea* L. cv Pusa Bold, *B. carinata* cv Pusa Gaurav (DLSC 1) and *B. napus* var Neelam (HPN-3). Correlation amongst the different parameters tested for screening salinity tolerant was also studied. In the present investigation, growth, chlorophyll and protein content of the seedlings decreased sharply in all the species upon salinity treatment. Electrolyte leakage analysis indicated that membrane damage of *B. juncea* seedlings was least whereas endogenous K^+/Na^+ ratio was found to be higher. Strong positive correlation between the electrolyte leakage and malondialdehyde content analysis has been obtained ($r=0.9$). The response of all the three amphiploid *Brassica* species under salinity condition differed significantly ($p \leq 0.01$). Amongst the three amphiploid species *B. juncea* L. cv Pusa Bold was found to be more tolerant.

Keywords: Abiotic Stress; Brassica; Electrolyte Leakage; Lipid Peroxidation; Proline.

Introduction

Plants exposed to the natural environment generally encounter various abiotic stresses which includes low and high temperature, drought, salinity, or the biotic stress like viruses, insects, nematodes, bacteria, fungi etc. These stresses greatly affect the plant productivity and it has been estimated that almost 50% of the crop yield is reduced due to abiotic stress whereas around 20-30% by biotic stress [1]. Amongst the various abiotic stresses soil salinity greatly affect the crop productivity [2].

In the past several efforts have been made for the development abiotic stress tolerance crop using transgenic technology but only little success has been achieved owing to its multigenic and quantitative nature [3]. Salinity tolerance is a complex process, it involves several physiological, molecular and biochemical process, and the level varies from species or amongst varieties within same species [4-8].

Under salinity stress, the morphological changes are the first response which can be seen very clearly [9]. However, these changes may not be enough to differentiate amongst species or varieties within same species. It is important to investigate the physiological, biochemical or molecular changes such as relative water content, toxic ions, osmotic potential,

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photosynthetic pigment, activity of enzymes, proteins/genes expression under salinity condition in order to understand the mechanism of salinity tolerance in plants [10-13].

One of the methods for understanding the mechanism and developing salinity tolerance in crop plants is by screening of available exotic cultivars of crop plants for salinity tolerance [14-17]. It has two major advantages, ûrst the tolerant genotype thus made available can be used in breeding programs and second, a comparative analysis at physiological/biochemical and/or molecular level of these contrasting cultivars can help us in understanding and unraveling novel survival mechanisms [18,19].

Brassica occupy third place among the various oilseed species but their growth, yield, and oil production are markedly reduced due to salinity. There is significant inter and intraspecific variation for salt tolerance within *Brassica* species which includes both amphidiploids and diploid species [20, 21]. An amphidiploid species *Brassica napus* (AC genome, n = 19) is derived from hybridization between *B. rapa* (A genome, n = 10) and *B. oleracea* (C genome, n = 9), *B. juncea* derived from *B. rapa* (A genome, n = 10) and *B. nigra* (B genome, n = 8) and *B. carinata* derived from *B. oleracea* (C genome, n = 9) and *B. rapa* (A genome, n = 10) [22].

Since the amphidiploid species were derived from diploid species it can be expected that the amphidiploid will have traits from both the parents and tolerate salinity much better than diploid species [23]. Therefore, the present investigation was carried out in order to determine variations in degree of salt tolerance amongst amphidiploid *Brassica* at the seedling stages.

Materials and Methods

Plant Material and Germination

Seeds of three different amphidiploids *Brassica* species i.e., *B. juncea* L. cv Pusa Bold, *B. carinata* cv Pusa Gaurav (DLSC 1) and *B. napus* var Neelam (HPN-3) were procured from Indian Agricultural Research Institute (IARI), New Delhi. Seeds were washed with de-ionized water and surface sterilized with 0.1% $HgCl_2$ and Bevastin. Seeds were allowed to germinate in a hydroponic system for 48 h in dark and then transferred to light for further growth under control conditions (25 ± 2^0C, 12 h light and dark cycles) in plant growth chamber.

Salinity Stress Treatment

For salinity stress treatment, 7 days old seedlings were treated with 200 mM NaCl for 24 h using hydroponic system. Simultaneously, seedlings maintained in de-ionized water were taken as control. After stress treatment, seedlings were harvested for growth analysis, electrolyte leakage analysis, Na^+ and K^+ estimation, chlorophyll assay, MDA assay, protein content analysis and proline assay.

Growth Analysis

To study the effect of salinity stress on seedling growth after 24 h of salt treatment, the root and shoot length of the seedlings were compared with unstressed control seedlings. Since the various species analyzed in this study had different rates of growth under control conditions, comparison of these species was based on the relative percentage change which was calculated by applying the formula [(Control – stressed)/ Control] x 100.

Electrolyte Leakage

Membrane damage due to salinity stress was evaluated by measuring electrolyte leakage as previously described [24]. After 24 h, stressed as well as unstressed seedlings were harvested and washed with distilled water to remove surface ions. Around 100 mg tissue was dipped in 20 ml of distilled water and incubated at 32°C for 2h. The initial electrical conductivity (E1) of the immersion solution was measured using conductivity meter (Ri Digital Conductivity Meter, Model 215-R). Then the seedlings along with immersion solution were autoclaved for 15 min at 121°C, cooled, and final electrical conductivity (E2) was measured. The relative electrical conductivity was calculated by the formula (E1/E2) x100.

Estimation of Na^+ and K^+ Contents

Determination of endogenous Na^+ and K^+ contents was done using Flame photometer following the protocol previously described [24]. Around 100 mg of seedling tissue (unstressed or salinity stressed) of each of the three *Brassica* species were predigested by soaking overnight with 10 ml of concentrated HNO_3 and finally digested with di-acid mixture (20 ml) containing HNO_3 and $HClO_4$ acid (9:4). The digested material was cooled, diluted with distilled water and filtered through Whatman No. 42 filter paper. The volume of the ûltered extract was made upto 30 ml with distilled water and was used to measure specificions.

Chlorophyll Estimation

Unstressed and stressed seedlings were harvested and approximately 50 mg leaves were used for the extraction. Chlorophyll pigments were extracted by immersing leaves in tube containing 1 ml of 80% acetone for 12 h in the dark at 4^0C. Supernatant were obtained after centrifugation at 5000 g for 5 min at room temperature and absorbance was recorded at 663 nm and 645 nm using spectrophotometer (Spectra Max M2). The amount of chlorophyll a and chlorophyll b was calculated according to the protocol described by Arnon [25]. The relative percentage decline in chlorophyll a and chlorophyll b was calculated by applying the formula [(Control – stressed)/ Control] x 100.

Total Protein Content Analysis

Salinity stressed and unstressed seedlings were harvested in liquid nitrogen and grinded to powder in mortar and pestle. Protein was extracted using phosphate extraction buffer (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, d.H_2O, pH 7.2). Supernatant obtained after centrifugation at 9000 g at 4^0C for 15 minutes was used for estimation by Bradford assay [26]. Concentration of total protein in the extract was estimated by measuring the absorbance at OD_{595nm} using spectrophotometer (Spectra Max M2).

Malondialdehyde Estimation using TBARS Assay

Malondialdehyde **(**MDA) was extracted from approximately 50 mg seedlings (unstressed and stressed) using 1 ml 0.25% Thiobarbituric acid (TBA) dissolved in 10% trichloroacetic acid [27]. Tube containing extract was incubated at 85°C for 30 min and then immediately chilled on ice. Supernatant was obtained after centrifugation at 10,000 g for 10 min and absorbance was recorded at 532 nm (MDA-TBA complex) and 600 nm spectrophotometer (Spectra Max M2). The OD_{600} values were subtracted from MDA-TBA complex values at 532 nm and MDA concentration was calculated using the Lambert-Beer law with an extinction coefficient εM =155/mM/cm. The level of lipid peroxidation is expressed as μM MDA/g.

Proline Estimation

Proline was extracted from both stressed and unstressed seedlings following the protocol previously described [28]. Around 100 mg seedlings were homogenized in 5 ml of 3% (w/v) aqueous sulphosalicylic acid and centrifuged at 12 000 g for 10 min to obtain supernatant. The reaction mixture (1 ml supernatant: 1 ml acid-ninhydrin : 1 ml glacial acetic acid) was incubated at 100°C for 1 h and terminated immediately by transferring to ice bath. The reaction mixture was extracted with 2 ml Toluene, mixed vigorously and allowed to cool down at room temperature for 30 min until separation of the two phases. The optical density of an upper phase was measured at 520 nm using toluene for a blank. The proline content was determined from a standard curve using pure proline.

Statistical Analysis

All experiments were repeated three times. For each experiment at least 10 seedlings were taken. Results are presented as mean ± S.E. Analysis of variance of data and their correlation between different parameters were calculated using the built-in data Analysis ToolPak in MS Excel.

Results

Effect of Salinity Stress on Seedling Growth

Upon 200 mM NaCl treatment, seedlings of *B. juncea* L. cv Pusa Bold, *B. carinata* cv Pusa Gaurav (DLSC 1) and *B. napus* var Neelam (HPN-3) responded differently (Figure 1). Under the imposed of 200 mM NaCl stress treatment for 24 h seedlings of all the cultivars lost their turgidity and color of leaves turned yellow and tip turned pale. All the species differed significantly in growth in response to 200mM NaCl for 24 h ($p \leq 0.01$). The growth of all the *Brassica* species reduced when compared to unstressed control (Figure 1). The seedlings showed reduction in both root and shoot length upon 24h of salinity stress. In order to study the effect of salinity stress on reduction in root and shoot length of seedlings the relative percentage reduction were calculated. The relative percentage reduction in root length was found to be 10.2%, 18.99% and 18.90% for *B. juncea B. carinata* and *B. napus,* respectively (Figure 2a). Similarly, the relative percentage reduction in shoot length was found to be 10.44%, 28.35% and 17.06% for *B. juncea B. carinata* and *B. napus,* respectively (Figure 2b). Stress treatment caused excessive wilting of seedlings of *B. carinata* and *B. napus* in compare to *B. juncea.*

Effect of NaCl on Membrane Stability

Stability of cell membrane significantly differed amongst cultivars, in response to 200mM NaCl for 24 h ($p \leq 0.01$). Electrolyte leakage in seedlings of *Brassica carinata* was found to be very high (82%) compared to

other two *Brassica* species (Figure 2c). *Brassica juncea* exhibited a minimum electrolyte leakage of 53% whereas it was 65% for *Brassica napus*. This analysis clearly indicates that cell-membrane stability was least affected under salinity stress in seedlings of *Brassica juncea.*

Effects of Salinity on Cytosolic Na^+ and K^+ Content

In the present investigation endogenous Na^+ and K^+ content was estimated in salinity stressed seedlings. Endogenous Na^+ and K^+ content differed significantly amongst *Brassica* species ($p \leq 0.01$). Accumulation of Na^+ content was found to be 1.8, 4.2 and 3.5 mg g^{-1} FW for *B. juncea B. carinata* and *B. napus,* respectively. All the *Brassica* species maintained high K^+ content which was found 8.4, 7.6 and 7.4 mg g^{-1} FW for *B. juncea B. carinata* and *B. napus,* respectively. It was observed that *Brassica juncea* maintained high K^+/Na^+ ratio as compared to other two *Brassica* species (Figure 2d).

Effects of Salinity on Chlorophyll Content

Salinity induced decline in chlorophyll content was observed in all the *Brassica* species. Under salinity stress treatment chlorophyll a content was found to be 0.38, 0.24 and 0.14 mg g^{-1} DW compared to the unstressed control value of 0.47, 0.35 and 0.38 mg g^{-1} DW resulting in chlorophyll a reduction of 19.14%, 31.42% and 63.15% in *B. juncea*, *B. napus* and *B. carinata,* respectively (Figure 2e). Similarly, percentage decline in chlorophyll b content was also calculated. Under salinity stress, chlorophyll b content was found to be 0.19, 0.12 and 0.09 mg g^{-1} DW compared to the unstressed control value of 0.3, 0.18 and 0.21 mg g^{-1} DW resulting in chlorophyll b reduction of 36.66%, 33.33% and 57.14% in *B. juncea*, *B. napus* and *B. carinata,* respectively. Thus, the percentage decline in chlorophyll a and chlorophyll b content was maximum in *B. carinata* and least in *B. juncea.*

Effects of Salinity on Total Protein Content

Under salinity stress treatment protein content decreased in all the *Brassica* species. It was noted that under salinity stress *B. juncea* maintained higher protein content than *B. carinata* and *B. napus*. Protein content under salinity stress decreased significantly by 13.62%, 22.60% in *B. juncea*, *B. napus* and maximum of 57.21% in *B. carinata,* respectively (Figure 2f).

Effects of Salinity on Lipid Peroxidation

The lipid peroxidation was measured by determining the level of malondialdehyde (MDA) as indicator of lipid peroxidation. MDA content was increased under salinity stress treatment for 24 h in all the *Brassica* species. Percentage increase in MDA content was 20%, 31.69% and 62.4% for *B. juncea*, *B. napus* and *B. carinata,* respectively (Figure 2g).

Effects of Salinity on Proline Content

Under salinity stress treatment for 24 h, accumulation of proline amongst *Brassica* species differed significantly ($p \leq 0.01$). Proline content accumulation was found to be 0.66, 0.43 and 0.21 mg g^{-1} DW in *B. juncea*, *B. napus* and *B. carinata,* respectively. Percentage increase in proline content was 86.3%, 81.7% and 71.8% for *B. juncea*, *B. napus* and *B. carinata,* respectively (Figure 2h).

Fig. 1: Effect of 200 mM NaCl for 24h in the Brassica seedlings. (1) *B. juncea* unstressed control, (2) *B. juncea* stressed, (3) *B. carinata* unstressed control, (4) *B. carinata* stressed, (5) *B. napus* unstressed control, (6) *B. napus* stressed.

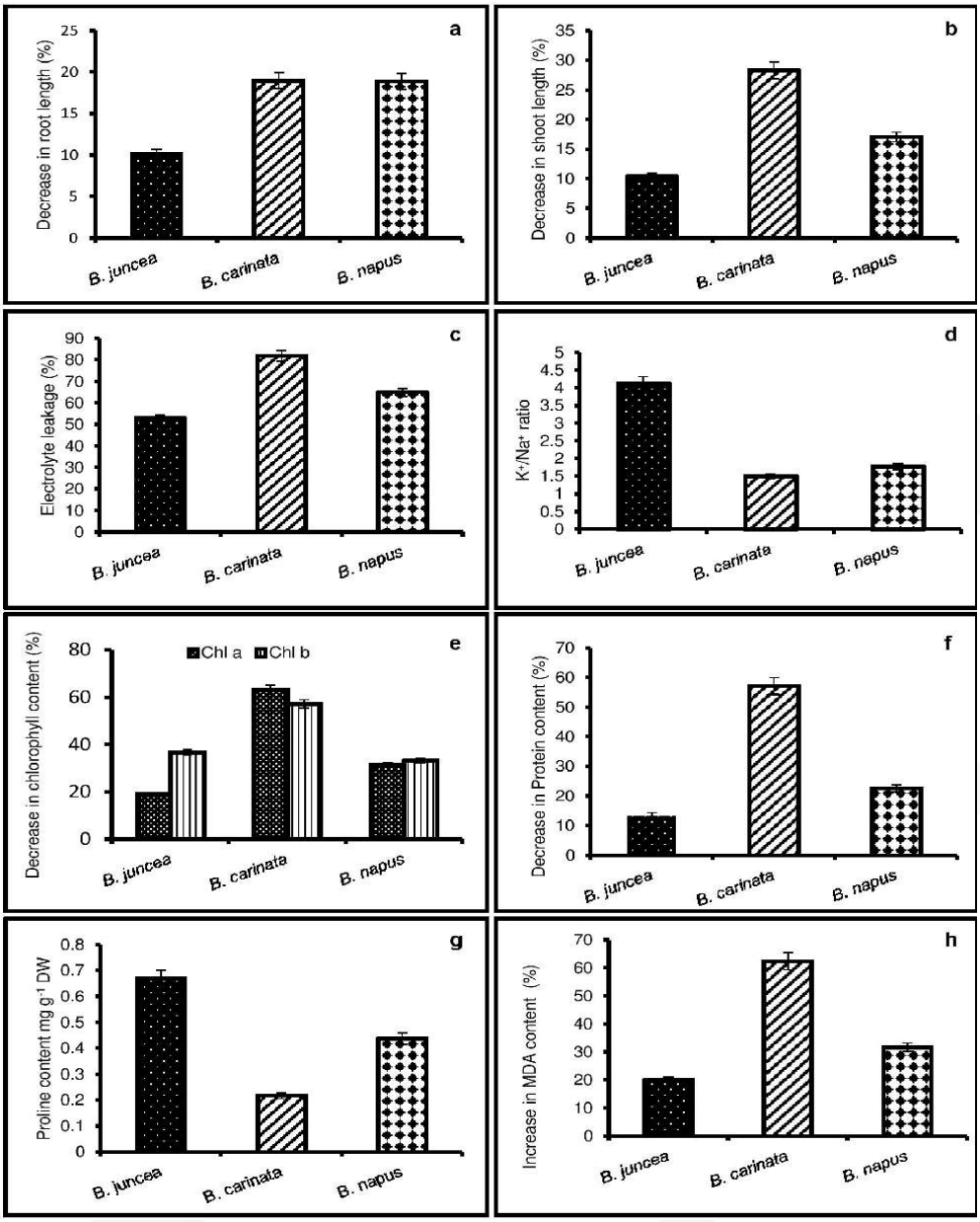

Fig. 2: Analysis of various morphological and biochemical parameters in the Brassica seedlings after 200mM NaCl treatment for 24 h. (a) Relative percentage decrease in root length. (b) Relative percentage decrease in shoot length. (c) Percentage change in electrolyte leakage. (d) Ratio of K^+/Na^+. (e) Relative percentage decrease in chlorophyll content. (f) Relative percentage decrease in protein content. (g) Endogenous proline content (mg g^{-1}DW). (h) Relative percentage increase in MDA content.

Discussion

Soil salinity is a major factor that reduces the productivity of crop worldwide [29]. Soil salinity is caused may be due to two reason; firstly, due to high rate of evapotranspiration and secondly, when leaching of the inorganic salts from the soil surface is very less resulting in the increase of soil salinity and sodicity [30]. The use of poor quality irrigation water is also the greatest cause of salinity [3]. Any change in soil condition can easily be sensed first of all by root organ system and the message is then conveyed to other parts of the plant like the tissues in shoot and leaves [31]. In the present study, decrease in root and shoot length were observed when seedlings were grown under salinity condition as observed in the decrease in plant length when treated with 200 mM NaCl. Reduction of plant growth by salinity differs between species and even between varieties and cultivars due to variability of salt tolerance among domestic and wild germplasms [7]. In the present

study, all the *Brassica* species responded differently upon salinity treatment. The relative percentage decrease in growth was found maximum for *B. carinata* cv Pusa Gaurav (DLSC 1) compare to other two amphidiploids species. Reduction in plant growth is due to reduction in the osmotic potential that restricts the absorption of water and nutrients by roots [32]. Salinity stress causes accumulation of salt ions in cells that causes toxicity and this can clearly visible in plants by chlorosis and necrosis of the leaf tissues [33]. In the present study, salinity stress not only brought about change in the growth but also changed the color of leaves to yellow and lost turgidity which was very clear among all the *Brassica* cultivar compared to unstressed control. These changes are due to decrease in chlorophyll content and water content of cells, resulting reduction in photosynthetic activity and turgidity [34]. Many studies confirm the inhibitory effect of salinity on biochemical processes, of which photosynthesis is the most important [35]. Turgidity lost due to salinity stress may cause injury in the cell membrane. The technique for the estimating the membrane damage is measuring the solute or ions leached out from the cell upon injury. Electrolyte leakage measurement is the indication of amount cell injury or membrane damage. Integrity or stability of cell membrane may vary amongst species or varieties of same species. In the present study all the *Brassica* species responded differently under salinity stress. *Brassica juncea* cv. Pusa Bold exhibited a minimum electrolyte leakage compared to other two *Brassica* species which clearly indicates that cell-membrane stability was least affected under salinity stress. A robust membrane can selectively restrict the entry of Na^+ ions into cells which is one of the key features of plant salt tolerance thereby maintaining the optimal K^+/Na^+ ratio in the cytosol [36]. Under salinity conditions, absorption of Na^+ and Cl^- competes with nutritional elements such as K^+, N, P, and Ca^{2+} by plants, resulting in ionic imbalance in the cell [37]. More Na^+ enters the cell due to similar in the hydrated ionic radii between Na^+ and K^+ makes it difficult for the transporter to discriminate between the two ions [38, 39]. However, tolerant plants overcome restricting the entry of Na^+ ions into cells, extrusion of Na^+ ions out of the cell or/and vacuolar compartmentation of Na^+ ions. In the present study, *B. juncea* cv. Pusa Bold maintained high ratio of K^+/Na^+ ratio compared to other two *Brassica* species possibly exhibiting combination of these strategies and is hence able to maintain favourable K^+/Na^+ ratio. Several proteins or transporter have been reported to play important role in pumping out excess of Na^+ ions out of the cell in tolerant plant.

Lipid peroxidation has been associated with cell damages caused by different biotic and abiotic stresses and is often used as an indicator of salt-induced oxidative damage to the cellular membranes [40]. Therefore in the present study, lipid peroxidation was measured by determining the level of malondialdehyde (MDA) content. There was a strong positive correlation between the electrolyte leakage and MDA content under salinity stress (r=0.9). The species showed maximum electrolyte leakage had higher MDA content. However, salinity stress may have positive or negative effect on protein content. In the present investigation, under salinity stress treatment protein content decreased in all the *Brassica* species. Excess of Na^+ content in the cell may degrade enzymes/proteins thereby affecting the whole biochemical and cellular process. Protein content was found to be higher in *B. juncea* compare to *B. carinata* cv Pusa Gaurav (DLSC 1) and *B.napus* var Neelam (HPN-3) indicating that *B. juncea* cv. Pusa Bold has better mechanism for overcome NaCl stress than other two species. Tolerant plant tries to maintain higher protein content because proteins serve as a reservoir of energy or may be adjuster of osmotic potential in plants subjected to salinity [41]. Under salinity stress, level of total free amino acids was reported to be higher in the leaves of salt tolerant in compare salt sensitive lines of sunflower, *Eruca sativa* and *Lens culinaris* [42-44]. Many amino acids including proline, alanine, arginine, glycine, serine, leucine, and valine and the non-protein amino acids and amides accumulate in plants exposed to salt stress [41]. Proline is a major amino acid that accumulates in the cytosol during salinity stress and accomplished osmotic adjustment [45-47].

Conclusion

Salinity tolerance is a complex process it is a cumulative effect of responses at physiological, molecular and biochemical levels. Therefore, in order to understand the molecular mechanism of salinity tolerance in plants it is important to investigate different parameters like RWC, ions contents, photosynthetic pigment, cell membrane injury, activity of enzymes, proteins/genes expression under salinity condition. In the present investigation, *B. juncea* L. cv Pusa Bold tolerated salinity better than other two amphidiploids *Brassica* species i.e., *B. carinata* cv Pusa Gaurav (DLSC 1) and *B. napus* var Neelam (HPN-3). Screening of available local/exotic cultivars and comparing them at physiological/ biochemical and/or molecular level will help us in understanding and unraveling novel survival mechanisms.

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