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Effects of Lignosulfonates on Callus Proliferation and Shoot Induction of Recalcitrant *Indica* Rice

(Kesan Lignosulfonat ke atas Proliferasi Kalus dan Induksi Tunas Beras *Indica* Rekalsitran)

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ABSTRACT

In vitro *culture of recalcitrant* indica *rice cultivar through intervening callus is difficult due to long regeneration period. Therefore, this study was undertaken to evaluate the growth promoting effects of lignosulfonate (LS) on callus proliferation and shoot induction of Malaysian recalcitrant* indica *rice cv. MR219. LS is a by-product of wood industry, commonly used as a plant growth enhancer. Seed derived calli were proliferated on Murashige and Skoog (MS) medium supplemented with different ion-chelated LS (calcium LS: CaLS and sodium LS: NaLS) at 50, 100, 150, and 200 mg/L. MS supplemented with 100 mg/L CaLS significantly increased the callus proliferation rate and adventitious root formation. In shoot induction study, both LSs did not enhance the shoot induction efficiency as compared to the control. However, the formation of albino shoot increased in MS fortified with 100 mg/L CaLS. Further chlorophyll and molecular analyses showed that, albino shoots induced from 100 mg/L CaLS had severe reduction in total chlorophyll content and expression of both chlorophyll-associated genes,* chlorophyll a/b-binding protein 1 *(OsCAB1R) and* young seedling albino *(OsYSA). Taken together, LS improves callus proliferation rate and modulate different physiological responses during plant growth of recalcitrant* indica *rice.*

Keywords: Albino; callus proliferation; indica *cv. MR219; lignosulfonate; regeneration*

ABSTRAK

Pengkulturan kultivar beras indica *rekalsitran secara* in vitro *melalui kalus intervensi adalah sukar kerana tempoh regenerasinya yang panjang. Oleh itu, kajian ini dijalankan untuk menilai kesan lignosulfonat (LS) terhadap proliferasi kalus dan induksi tunas beras* indica *rekalsitran Malaysia cv. MR219. LS adalah produk sampingan daripada industri kayu yang biasanya digunakan sebagai perangsang pertumbuhan tumbuhan dalam baja. Kalus diperoleh daripada biji diproliferasi atas medium Murashige dan Skoog (MS) yang ditambah dengan pengikat ion LS (kalsium LS: CaLS dan natrium LS: NaLS) pada kepekatan 50, 100, 150 dan 200 mg/L. MS yang ditambah dengan 100 mg/L CaLS didapati meningkatkan kadar perkembangan kalus dan pembentukan akar serabut. Dalam kajian induksi tunas, kedua-dua LSs tidak meningkatkan kecekapan induksi berbanding dengan kawalan. Walau bagaimanapun, penghasilan albino meningkat pada MS yang ditambah dengan 100 mg/L CaLS. Analisis klorofil dan molekul menunjukkan bahawa albino yang diinduksi daripada 100 mg/L CaLS mempunyai pengurangan yang banyak dalam jumlah kandungan klorofil dan pengekspresan kedua-dua gen yang berkaitan dengan klorofil,* chlorophyll a/b-binding protein 1 *(OsCAB1R) dan* young seedling albino *(OsYSA). Sebagai kesimpulan, LS meningkatkan kadar proliferasi kalus dan memodulasi tindak balas fisiologi yang berlainan semasa pertumbuhan beras* indica *rekalsitran.*

Kata kunci: Albino; indica *cv. MR219; lignosulfonat; proliferasi kalus; regenerasi*

INTRODUCTION

Rice is one of the major staple foods in the world. In 2016, the rice production of Malaysia was recorded at 3 million tons (FAO 2017). However, the current rice production is not sufficient to sustain the local domestic consumption requirements where Malaysia is still largely dependent on imported rice. Thus, to fulfill the local rice demand, research in rice has been generally focusing on improving the grain yield and quality. The *Oryza sativa indica* cv. MR219 is an important local rice cultivar with high yield and largely cultivated in Malaysia (FFTC 2002).

To date, major achievement in genetic manipulation of rice has been obtained mostly in *japonica* rice because of its better response to *in vitro* culture as compare to other cultivars (Andrew Kok et al. 2018; Visarada & Sarma 2004). Nevertheless, crop improvement based on genetic manipulation on *indica* rice remains the major limiting factor due to its recalcitrant characteristics which include low regeneration rate, low transformation efficiency and as well as long regeneration period (Lai et al. 2011; Martinez-Trujillo et al. 2003; Mishra & Rao 2016; Raghavendra et al. 2010; Sah et al. 2014; Sahoo et al. 2011). Low regeneration

rate had been observed in previous studies whereby the regeneration efficiency was around 7% (Abiri et al. 2017) and 13% in MR219 (Htwe et al. 2011). Long embryogenic callus formation period was also observed in MR219 whereby 8 weeks was required for embryogenic callus formation prior to shoot regeneration (Zuraida et al. 2012). Hence, effective culture mediums for callus proliferation and shoot induction are prerequisite to ensure the success of genetic manipulation for traits improvement in recalcitrant *indica* rice.

Lignosulfonate (LS) is a sulfonated polymer arising from three phenylpropanoid monomers mainly, coniferyl, sinapyl and p-coumaryl alcohol. This complex polymer is a low-cost waste product from sulfite pulping process in wood industries that is already being commercialized as binding agent and dispersal for several industrial purposes (Almas et al. 2014; Yang et al. 2007). Commercial LS usually comes in different cation-chelated such as Ca, Na, Zn, K or Fe, depending on the type of sulfite used in pulping process. In agriculture, the ability to chelate different micronutrient ions had made LS one of the ideal compositions in plant fertilizer, besides being cost-effective as compared to other additives (Carrasco et al. 2012). It has also drawn considerable interest to be used as stimulant in plant growth and fructification (Ertani et al. 2011; Rodríguez-Lucena et al. 2009; Telysheva et al. 1997, 1992; van der Krieken et al. 2004). Additionally, LS also significantly enhances the rooting and shoot growth of ornamental plants (Telysheva et al. 1997, 1992).

Therefore, the present study was undertaken to extend *in vitro* application of LS in enhancing callus proliferation and shoot induction of recalcitrant Malaysian *indica* rice cv. MR219. Ultimately, we hope to improve the cultivation medium of this local recalcitrant *indica* rice through the supplementation of LS.

Materials and Methods

PLANT MATERIALS

The recalcitrant Malaysian rice (*Oryza sativa* L. ssp. *indica* cv. MR219) seeds used in this study were obtained from Malaysian Agricultural Research and Development Institute (MARDI), Seberang Prai, Penang.

CHEMICALS

Four different types of LS chosen were: industrial grade calcium lignosulfonate (iCaLS) and sodium lignosulfonate (iNaLS) from Borregaard, Norway; analytical grade calcium lignosulfonate (aCaLS) and sodium lignosulfonate (aNaLS) from Sigma, USA.

SEEDS STERILIZATION

The mature seeds were sterilized according to previously described protocol (Lim & Lai 2017) with slight modifications. Briefly, seeds were dehusked and rinsed with sterile distilled water. Then, the seeds were surface-sterilized with 70% (v/v) ethanol for 1 min and 50% (v/v) Clorox for 30 min, followed by washing 4-5 times with sterile distilled water. Sterile seeds were dried before use.

CALLUS INDUCTION AND PROLIFERATION

Gamborg's B5 basal medium (Gamborg et al. 1968) supplemented with 10 g/L maltose, 10 mg/L NAA and 1 mg/L 2,4-D was employed to induce calli from sterile mature seeds in darkness for two weeks at 25 ± 2°C. For callus proliferation study, 50 mg of 2-week old callus clump was cultured on Murashige and Skoog (MS) medium (Murashige & Skoog 1962) containing 2 mg/L 2,4-D and 0.5 mg/L kinetin with different types and concentrations (50, 100, 150, and 200 mg/L) of LS. LS was excluded in control treatment. Each petri dish contained nine callus clumps. The fresh weight (FW) and dry weight (DW) of the calli were measured after three weeks of cell proliferation. The FW of calli were measured directly before subjecting the calli for drying in oven at 70°C. DW of the callus measurement were taken weekly until constant DW is obtained. Number of calli forming adventitious root in the control and optimum LS condition was also recorded. Experiments were performed in triplicates with three petri dishes per replicate.

SHOOT INDUCTION

MS medium containing 30 g/L sucrose and 3 mg/L kinetin supplemented with different types and concentrations (100, 200, 300, 400, and 500 mg/L) of LS were used in shoot induction study. Meanwhile, control treatment had no LS. One-month old proliferated callus clumps were cultured in shoot induction medium under a photoperiod for 16 h light and 8 h dark at 25 ± 2°C. Number of shoots induced and the morphology changes were recorded after two months of *in vitro* cultivation. Experiments were performed in triplicates with 10 callus clumps per replicate.

DETERMINATION OF TOTAL CHLOROPHYLL CONTENT

To further confirm and measure the level of chlorophyll in induced albino shoot, chlorophyll content determination was performed according to protocol described by Yap and Lai (2017) with slight modifications. Fresh leaves (200 mg) from the *in vitro* culture were ground in liquid nitrogen and homogenized in 5 mL of 80% (v/v) acetone followed by centrifugation at 3000 × g for 20 min. The pigment content was analyzed with UV/Vis spectrophotometer at the wavelengths of 663 and 645 nm. The total chlorophyll, chlorophylls a and b in each sample were determined using the Arnon's equation (1949): Chlorophyll a (μg/mL) = $12.7(A_{663}) - 2.69(A_{645})$; Chlorophyll b (μg/mL) = $22.9(A_{645}) - 4.68(A_{663})$ and total chlorophyll (μg/mL) = $20.2(A_{645}) + 8.02(A_{663})$.

TOTAL RNA ISOLATION

The total RNA of wild type (control) and albino shoot induced from 100 mg/L aCaLS medium was isolated using the RNeasy Mini Kit (Qiagen). Quality and purity of total RNA were measured using the Thermo Scientific™ NanoDrop 2000. The absorbance 260/280 nm of RNA was 1.8-2.0. Then, 1 ug of total RNA was employed for first strand cDNA synthesized using QuantiNova reverse transcription kit (Qiagen).

REAL-TIME REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (QRT-PCR) ANALYSIS

The chlorophyll-associated gene specific primers used were: forward 5' AGA TGG GTT TAG TGC GAC GAG 3' and reverse 5' TTT GGG ATC GAG GGA GTA TTT 3' for *OsCAB1R*; forward 5' CAG GAG ATT GCC GAT GGG TT 3' and reverse 5' TCG ATT CCC CGT GCA TAA GG 3' for *OsYSA*. The internal controls used for expression normalization were rice *ubiquitin 5* (*OsUBQ5*) and *elongation factor 1 alpha* (*OsEF1α*) genes. The primers for these genes were: forward 5' TAG GCG TAG GCT CCT GTT CT 3' and reverse 5' ACA GAG GTG ATG CTA AGG TGT 3' for *OsUBQ5*; forward 5' ACC AGA TCA ACG AGC CCA AG 3' and reverse 5' CTC CAG TCT CAA CAC GAC CC 3' for *OsEF1α*. Real-time PCR analysis was conducted according to previously described protocol with slight modifications (Lai & Takehisa 2013; Lai et al. 2012). Briefly, the analysis was performed using the Bio-Rad CFX96 system with SsoFast™ EvaGreen® Supermix (Bio-Rad). The qPCR profile was: Enzyme activation at 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 5 s. Three technical replicates with three biological replicates each were performed for each sample. Efficiency-corrected ΔC_T and $\Delta\Delta C_T$ were used to calculate the relative differences in transcript expression according to Livak's method (Livak & Schmittgen 2001).

STATISTICAL ANALYSIS

All data presented were the average ± standard deviation (SD) of three biological replicates. The analysis of variance (ANOVA) was used in evaluating the level of significant differences at $p<0.05$ between the different conditions using the SPSS v.20 software (IBM Corp., Armonk, USA).

RESULTS

After three weeks of callus proliferation on medium supplemented with different types and concentrations of LS, callus FW and DW increments were observed in all the treatments except for iNaLS. The optimum callus proliferation rate was recorded at 100 mg/L aCaLS with 88% FW (Figure 1(D)) and 32% DW increments as compared to the control (Figure 1(E)). In addition, adventitious root formation was also enhanced (1.4-fold) in treatment supplemented with 100 mg/L aCaLS (Figure 1(F)). Calli produced in all the LS treatment appeared to be yellowish resembling the embryogenic cell except in 200 mg/L iNaLS treatment, whereby browning of callus was observed (Figure 1(A)). The 100 mg/L aCaLS treated callus (Figure 1(C)) appeared to produce more adventitious roots in comparison to the control callus (Figure 1(B)). Moreover, both industrial and analytical grades of LS did not show significant different in their growth promoting effects.

Based on the results in Figure 2(E) and 2(F), successful shoot induction was only observed in MS medium supplemented with either 100 mg/L aCaLS or 200 mg/L aNaLS. However, half of the shoots induced from 100 mg/L aCaLS were found to be albino (Figure 2(D)) while none was observed in 200 mg/L of aNaLS. The wild type-liked shoots induced in 100 mg/L aCaLS were similar to the control (Figure 2(C)). In general, shoot induction efficiency was recorded lower in LS conditions as compared to the control. Besides, white microcalli were also observed in both LS and control cultures. Nevertheless, only white microcalli induced from control and media supplemented 100 mg/L aCaLS or 200 mg/L aNaLS were eventually regenerated into shoot. No sign of shoot regeneration was observed from the microcalli produced in other treatments. The highest microcallus production was obtained at 200 mg/L aNaLS and the lowest at 100 mg/L aNaLS (Figure 2(E) & 2(F)). Microcalli were friable and embryogenic with potential to differentiate into shoots (Figure 2(A) & 2(B)).

In addition, total chlorophyll content analysis was greatly reduced in albino shoots induced from 100 mg/L aCaLS treatment (Figure 2(G)). In accordance to low total chlorophyll content, transcription level of the two chlorophyll-associated genes namely, *chlorophyll a/b binding protein 1* (*OsCAB1R*) and *young seedling albino* (*OsYSA*), were also significantly down regulated in the albino shoots (Figure 2(H)). The *OsYSA* gene was not expressed, while *OsCAB1R* gene expression was severely reduced by 0.76-fold as compared to wild type.

DISCUSSION

Our study showed that aCaLS at concentration of 100 mg/L significantly increased the callus proliferation rate of MR219 rice. Similarly, addition of CaLS was also reported to enhance callus growth of *poplar* (Kevers et al. 1999), growth of *Phalaenopsis* and as well as rooting of *Sequoiadendron* (Docquier et al. 2007). According to Docquier et al. (2007), the effects of LS could be indirect or 'delayed' and they hypothesized that CaLS may regulate either hormonal changes, mineral balancing or regulates the numbers of auxin receptor and their affinity to free auxin. In general, micronutrient ions such as Fe, Zn and Mn are metal ions that could easily precipitate when there are changes in pH and chemical compositions. These solid complexes are not readily available to plant and thus, reduce the nutrient acquisition efficiency in plant. Therefore, LS could also act as a weak synthetic chelator that provides protection from

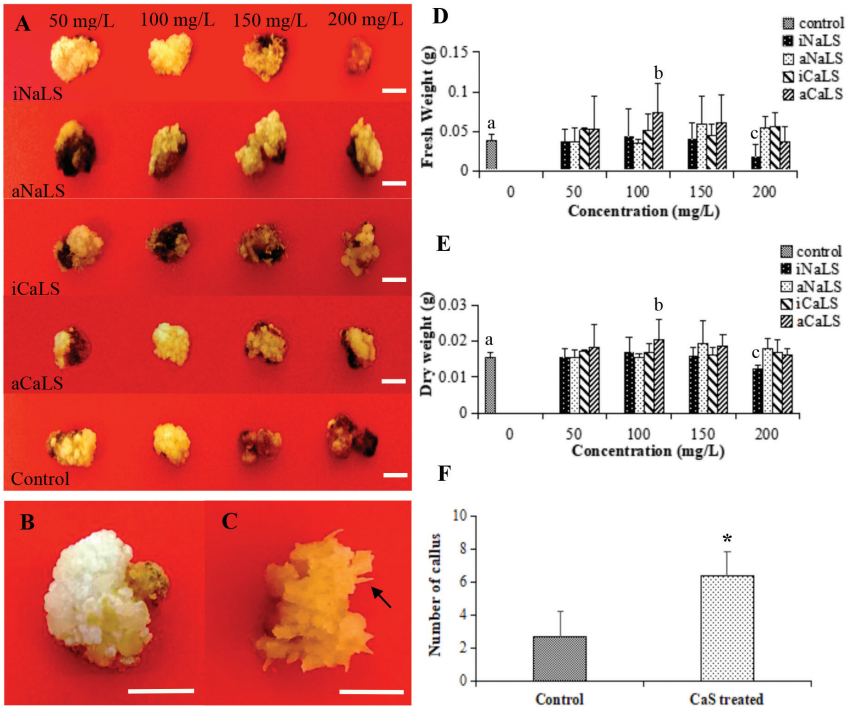

FIGURE 1. Callus proliferation treated with different types and concentrations of LS. (A) Morphology of callus, (B) Control callus, (C) 100 mg/L aCaLS-treated callus with adventitious root, (D & E) Mean of FW and DW of three weeks old calli and (F) Number of callus observed with adventitious root formation in control and 100 mg/L aCaLS treatment. Data shows mean of three biological replicates. Different alphabets represent the significantly difference between treatments at 5% level according to Duncan's Test. Asterisk indicates statistical significance at $p<0.05$. Arrow shows adventitious root. Scale bars represent 0.5 cm. Error bars represent standard deviation

insoluble complex formation and relatively fast release of the soluble micronutrient ions to cells for plant growth (Carrasco et al. 2012; Cieschi et al. 2016).

LS chelated calcium ions (Ca^{2+}) could prevent nutrient loss through precipitation due to pH changes or complex formation. Hence, availability of Ca^{2+} ions to plant in the medium increased. In general, Ca^{2+} is an essential micronutrient which plays a fundamental role as secondary signaling molecule in plant in response to environment stimuli (Tuteja & Mahajan 2007). The increased level of Ca^{2+} in plant cell triggered the calcium-binding protein (calmodulin) and consequently activated the downstream calcium-dependent protein kinases. These protein kinases then regulate the functions of various genes involved in cell division and elongation, cytoplasmic streaming and plant defense (DalCorso et al. 2010; Park et al. 2008; Yang & Poovaiah 2008). Hence, elevation of Ca^{2+} availability could be one of the major factors contributing to improve cell proliferation rate as seen in this study.

In shoot induction study, LS did not enhance the shoot induction efficiency as compared to control but a mixture of wild type and albino shoot populations were induced in aCaLS at 100 mg/L. This phenomenon could be caused by disrupted interaction between auxin and cytokinin. Auxin and cytokinin are the main plant hormones that regulate root and shoot development. In general, high ratio of auxin to cytokinin induces root formation while high ratio of cytokinin to auxin induces shoot regeneration (Schaller et al. 2015; Skoog & Miller 1957). Hence, we postulate that LS could be either elevating the endogenous auxin level that demotes shoot induction or depressing certain cytokinin transport proteins that affect the distribution of cytokinin throughout the cells.

To further understand the possible role of LS during shoot induction, gene expression analysis of chlorophyll-associated genes was performed. In plant, chlorophyll synthesis is controlled at multiple levels in both the nucleocytoplasmic and plastid compartments. The nuclear-encoded *OsCAB1R* gene encodes a light-harvesting *chlorophyll a/b-binding protein* of PSII, which are normally complexes with chlorophyll and xanthophylls and serve as the antenna complex to receive and transfer captured energy for the reaction centers of photosystem (Jansson 1999, 1994). Meanwhile, plastid-encoded *OsYSA* regulates the expression of genes related to chlorophyll biosynthesis, chloroplast development and photosynthesis in young seedling (Su et al. 2012). Our study demonstrated that the expression of both *OsCAB1R* and *OsYSA* genes was markedly reduced upon addition of 100 mg/L aCaLS during shoot induction. Furthermore, our results also imply that the occurrence of albino shoot induction is ion and concentration dependent. Hence, specific ion-chelated LS at optimum concentration may negatively regulate the transcript level of chlorophyll-associated genes, leading to chlorophyll synthesis disruption from both nucleocytoplamic and plastid levels.

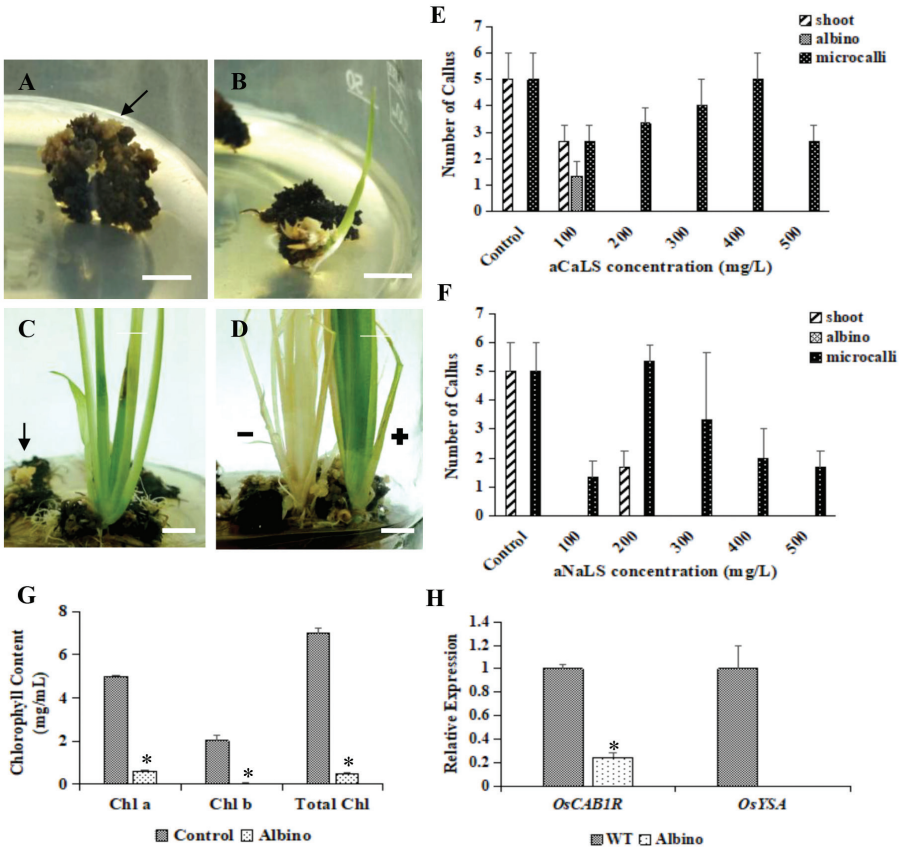

FIGURE 2. Shoot induction study treated with different types and concentrations of LS. (A & B) Stages in MR219 shoot induction from callus, (C) Wild type shoot induced in control, (D) Albino (-) and wild type-like (+) shoots induced in 100 mg/L aCaLS, (E) The number of shoots, albino and microcalli observed in different concentrations of aCaLS treatments, (F) The number of shoots, albino and microcalli observed in different concentrations of aNaLS treatments, (G) The total chlorophyll, chlorophyll a and b content in control and albino shoots and (H) Normalized relative expression of *OsCAB1R* and *OsYSA* in wild type (WT) and albino shoots. Data shows mean of three biological replicates. Asterisk indicates statistical significance at $p<0.05$. Arrows show microcalli. Scale bars represent 1 cm. Error bars represent standard deviation

To date, the molecular mechanisms underlying the growth enhancing responses induced by LS is remained largely unknown. In the past, LS was postulated to play a role in regulation of endogenous auxin concentration (Gaspar et al. 1996; Hausman et al. 1995), auxin protector from enzymatic degradation, increase auxin-tissue sensitivity (Telysheva et al. 1997, 1992) and as well as to facilitate the transfer of macro and micronutrients into the plant cell compartments (Carrasco et al. 2012; Cieschi et al. 2016; Yamashita 1996). Nevertheless, concerted and detailed experiments need to be carried out to further confirm these growth enhancing effects of LS during plant growth.

CONCLUSION

Taken together, LS improves callus proliferation rate and modulates different physiological responses during plant growth of recalcitrant *indica* rice. Results gathered from this study could be used to enhance *in vitro* culture of recalcitrant *indica* rice, particularly in callus proliferation rate that will shorten the duration of cultivation.

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