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Ozone Impacts on Soluble Carbohydrates, Antioxidant Activity and Macro-element Concentrations in Rice Seedling

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ABSTRACT: The present study describes carbohydrate metabolism, macro-element utilization and antioxidant defenses in response to an ozone dose (100 ppb, 8d) in two rice varieties. Tolerant (cv. Jinpumbyeo) and sensitive (cv. Chucheongbyeo) varieties of rice were grown in growth chamber for 30 days after sowing. Concentrations of chloroplast pigments and non-structural carbohydrates as well as activity of antioxidant enzymes were determined to evaluate the resistance against ozone stress. Ozone caused the decrease in chlorophyll a and carotenoid contents, and also resulted in faster decomposition of non-structural carbohydrate in leaf blade and leaf sheath. The contents of nitrogen and potassium in leaves were visibly decreased in cv. Chucheongbyeo with an increase in ozone exposure, but not in cv. Jinpumbyeo. Enzymatic antioxidants against ROS in both varieties responded in the order of POD, SOD and CAT, and their capacity was stronger in cv. Jinpumbyeo.

Keywords: non-structural carbohydrate, pigments, macro-elements, antioxidant, ozone, rice

Abbreviation : CAT, catalase; SOD, superoxide dismutase; POD, peroxidase; ROS, reactive oxygen species; PCA, perchloric acid; EDTA, ethylenediamine tetraacetate; PVP, polyvinylpyrrolidone; NBT, nitro blue teterzolium; BSA, bovine serum albumin; ICP, inducible coupled plazma;

In many advanced countries, ozone, is one of industrial pollutants, occurs high concentrations enough to induce physiological and biochemical changes at different plant levels. Peaks of high ozone concentrations (above 100 ppb) may often reach for short periods during the summer and may lead to visible foliar injury in sensitive plants.

Ozone can cause various adverse effects such as visible injuries, growth reductions, shifts in shoot : root biomass and the disturbance of normal physiological processes on plant species. Also, decrease in assimilation rate (Grulke *et al.*, 2002) and increase in respiration (Willenbrink & Schatten, 1993) can lead to less carbon fixation. Ozone can restrict phloem loading and thus the translocation of assimilates from shoots to root (Skärby *et al.*, 1998). This results in the decrease in storage compounds like soluble carbohydrates and starch especially in roots (Braun *et al.*, 2004). Most studies of the effects of ozone on the nutrient status of plants have been focused on the after anthesis. In snapbean (*Phaseolus vulgaris* L.), ozone fumigation decreased the concentrations of calcium, magnesium, iron and mangense in the leaves, but increased potassium, phosphorus and molybdenum concentrations in the pods (Tingey *et al.*, 1986). Fuhrer *et al.* (1990) reported that ozone exposure increased calcium, magnesium, potassium and phosphorus concentrations in the grain of spring. Additionally, plants exposed to ozone are limited in the use of mineral elements (K, Mg and Mn) which are essential for the functioning of stomata and enzymes central to carbon fixation. The phytotoxicity of ozone inside the leaves is probably due to its ability to react with apoplast constituents, thus generating highly reactive oxygen species (ROS), which include peroxides and free radicals which are the real cause of the negative effect of ozone (Hippeli & Elstner, 1996). In order to remove these toxic compounds, plants include both non-enzymatic compounds with high reducing potentials, such as ascorbic acid, β-carotene, polyamines and glutathione (Kangasjärvi *et al.*, 1994; Alscher & Hess, 1993) and enzymes such as ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR) and work together with the antioxidant scavengers. In addition to the ascorbate-glutathione cycle, superoxide dismutase (SOD), which convert the superoxide radical into hydrogen peroxide, as well as peroxidase (POD) and catalase (CAT) which metabolise the hydrogen peroxide to water play important roles in removing ROS (Kangasjärvi *et al.*,1994). In previous study, we classified 15 Korean rice varieties based on the

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ozone sensitivity, visible damage, at seedling stage (data not published). Of them, we took two rice varieties, ozone-tolerant (cv. Jimpumbyeo, below 20%) and ozone-sensitive (cv. Chucheongbyeo, over 40%). This study was performed to investigate the effects of ozone on photosynthetic products, macro-nutrients utilization and antioxidative capacity of two rice varieties differing in sensitivity to ozone.

MATERIALS AND METHODS

Plant materials and ozone fumigation

Seeds of two rice varieties (cvs. Jinpumbyeo and Chucheongbyeo) were germinated in plastic trays filled with soil and grown in a controlled environmental growth chamber at 25/20 °C (day/night), 70% RH, 300 μmol m^{-2} s^{-1} light and 12-h photoperiod. At 30 days after plants were sown, they were fumigated. Plants were exposed to 100 ± 10 ppb ozone for 8 days (4h d^{-1}). Immediately after 2, 4 and 8 days of fumigation, fully expanded leaves were randomly collected, frozen in liquid nitrogen and stored at - 70 °C for biochemical analyses.

Pigment determination

According to the method of Jeffrey & Humphrey (1975) and Strickland & Parsons (1972), total chlorophyll and carotenoid were assayed, respectively. Leaf pigment was extracted by homogenizing and boiling 0.5 g of fresh leaf samples in 20 ml of 95% ethanol. After centrifuged shortly, the content of chlorophyll and carotenoid were checked with spectrophotometer at 470, 644 and 648 nm and expressed as μg g^{-1} FW.

Non-structural carbohydrate determination

Soluble sugars were extracted by heating leaf samples in 80% ethanol, according to Roe method (1955). The alcoholic extract (1 ml) was mixed with 2 ml of fresh 0.2% anthorne in sulfuric acid (w/v) and the mixture was placed in boiling water for 10 min. After cooling on ice, the absorbance at 630 nm was measured. After the extraction of the soluble fractions, the solid residues were used for starch analysis. Starch was extracted two times with 9.3 N PCA. The starch concentration was determined by the same method as described above. Glucose was used as a standard for both soluble sugars and starch.

Macro-nutrients assay

The extraction and measurement of macro-nutrients were determined according to Walinga method (1989). The dried leaves were finely grounded with a ball-mill machine, incorporated with 3.3 ml of 368 mM of salicylic acid in 84.7% H_2SO_4 and stood for overnight. The decomposed samples were wet-digested at 300 °C for 3-4 h with adding some drops of 35% H_2O_2 until become to be colorless. Of extracts made up to 100 ml with dH_2O, one ml was mixed with 3 ml of reagent I composed of 100ml of 0.05% sodium nitroprusside dehydrate, 50ml of 3.19 M salicylate solution in 10 M NaOH and 5 ml of 4% EDTA, and 5 ml of reagent II including 200 ml of 75 mM disodium hydrogen phosphate dehydrate (pH 12.3) and 50 ml of 1% sodium hypochlorite. The absorbance of total nitrogen and phosphorus was measured at 660 and 880 nm, respectively. Ammonium sulphate and potassium hydrogen phosphate were used as a standard for analyzing total nitrogen and phosphorus, respectively. Potassium was measured at 770 nm with ICP using potassium nitrate as a standard.

Antioxidative enzymes activity

Fresh leaves (1g) were homogenized in 100 mM Na-phosphate buffer (pH 7.8) containing 0.1 mM EDTA, and 1% (w/v) PVP at 4 °C. The homogenates were centrifuged at 12,000 × g for 20 min, and supernatants were used for enzymes activity and protein content assay. All assays were done at 4 °C. Total soluble protein contents of the enzyme extracts were determined according to Bradford (1976) using BSA as a standard. SOD (E.C. 1.15.1.1) activity assay was performed with a slight modification of Beauchamp & Fridovich method (1971), which measures the inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm. One unit of enzyme activity was defined as the quantity of SOD required to produce a 50% inhibition of reduction of NBT and the specific enzyme activity was expressed as unit mg^{-1} protein g FW. Catalase (E.C. 1.11.1.6) activity was determined by monitoring the decomposition of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm following the method of Bergmeyer (1970). The reaction mixture contained 50mM potassium phosphate buffer (pH 7.0) and a proper amount of plant extract in a 3 ml. The reaction was initiated by adding 10 mM H_2O_2. The enzyme activity was defined as μmol H_2O_2 destroyed min^{-1} mg^{-1} protein g FW. Peroxidase (E.C. 1.11.1.7) activity was determined by monitoring the formation of guaiacol dehydrogenation product (extinction coefficient 6.39 mM cm^{-1}) at 436 nm followed by the method of Pütter (1974). Reaction mixture (3 ml) contained 100 mM potassium phosphate buffer (pH 7.0), 0.3 mM guaiacol and plant extract. The reaction was initiated by adding 0.1 mM H_2O_2. The enzyme activity is defined as μmol H_2O_2

destroyed min^{-1} mg^{-1} protein g FW.

Statistics

The experimental design was a randomized complete design with three replicates. All data were subjected to an analysis of variance and when significance ($P < 0.05$, 0.01, 0.001) occurred for the effect of variety and treatment, a least significant difference (LSD) was calculated using SAS 8.12.

RESULTS AND DISCUSSION

Chlorophyll a, chlorophyll b and carotenoid content showed different degrees of reduction depending on variety (Fig. 1). Ozone exposure exerted a significant ($p < 0.001$) impact on the chlorophyll a content of the leaves at 8 days of exposure, whereas ozone had no effect on chlorophyll b in either varieties. The ozone-induced decline in chlorophyll a content tended to proceed at a faster rate in 'Chucheongbyeo' compared with 'Jinpumbyeo'. As ever the chlorophyll a, the decline of the carotenoid content proceeded faster in 'Chucheongbyeo', which reducing at 2 days of ozone exposure. At the end of treatment, the carotenoid content for 'Jinpumbyeo' and 'Chucheongbyeo' decreased in 17% and 21% compared with the initiation time of treatment, respectively. Carotenoids play an essential role as antioxidant molecules, capable of scavenging the harmful singlet oxygen

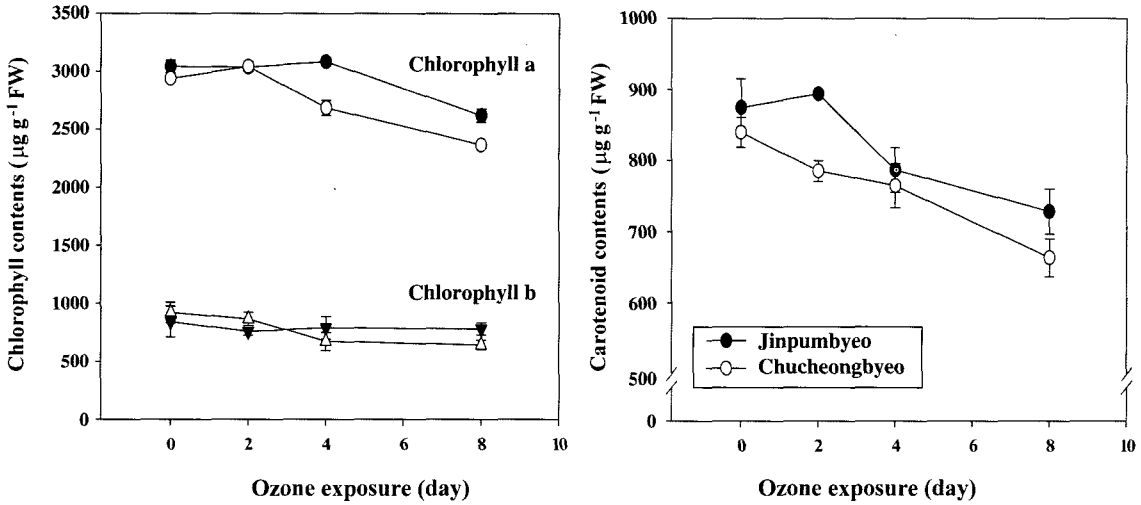

Fig. 1. Chlorophyll and carotenoid concentrations of two rice varieties. Rice seedlings were subjected to ozone (100 ± 10 ppb, 4 h) for 8 days. Symbols represent mean ± S.D. of three measurements. The statistical *p* values, chl a, chl b and carotenoid, of the variety effect are 0.001(***), 0.8032(ns) and 0.0198(*), respectively.

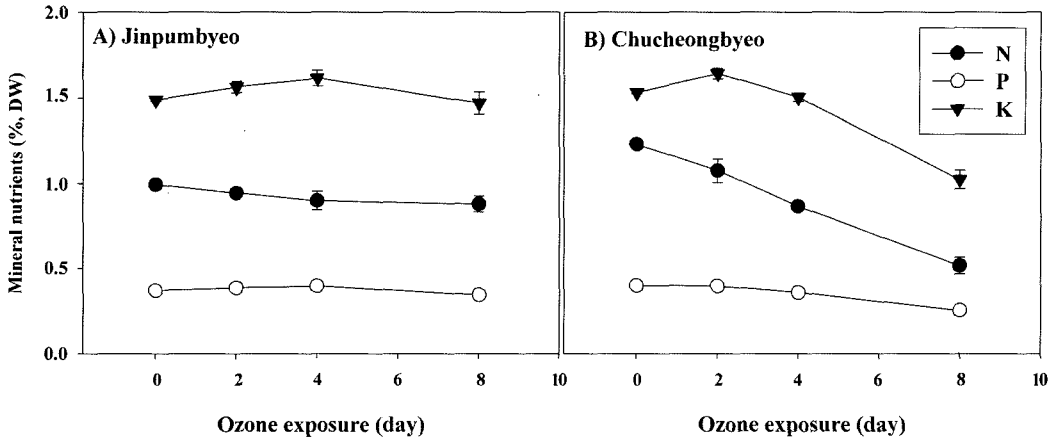

Fig. 2. Effect of ozone fumigation on nutrient concentrations in rice leaves. Rice seedlings were subjected to ozone (100 ± 10 ppb, 4 h) for 8 days. Symbols represent mean ± S.D. of three measurements. The statistical *p* values, nitrogen, phosphate and potassium, of the variety effect are 0.8029(ns), 0.0255(*) and 0.0033(**), respectively.

(Mikkelsen *et al.*, 1995) and of de-exciting chlorophyll a (Senser *et al.*, 1990). A decrease in chlorophyll and carotenoid can increase lipid peroxidation as much as the higher level of MDA under the ozone treatment. Alteration of pigment contents in many species under ozone exposure has been demonstrated by several researchers (Elvira *et al.*, 1998; Mikkelsen *et al.*, 1995) who reported that chlorophyll a and b is considered to be an ozone stress indicator. The present results showed difference in the sensitivity of chlorophyll a and carotenoid rather than chlorophyll b.

The macro-nutrient concentrations are given in Fig. 2. Ozone fumigation showed no effect on the nutrients in the leaves of cv. Jinpumbyeo. On the other hand, for cv. Chucheongbyeo, the concentrations of nitrogen and potassium were strongly reduced in proportion to the period of ozone fumigation. Ozone significantly affects nutrient status and relations for at least some nutrient elements. In our result, ozone interrupted the mobilization of nitrogen and potassium toward the leaves, and the magnesium concentration was also negatively influenced (data not shown), even though increases as well as decreases were stated for the individual nutrient concentrations (Utriainen *et al.*, 2001; Utriainen & Holopainen, 2001a). In accordance with Fuhrer *et al.* (1990) report, the ROS generated from ozone presumably decomposes the structure of chlorophyll molecules, as well as depresses the function of mineral nutrients (K, Mg, Mn) which are essential for stomata ability. Thereafter, the decrease in some nutrient concentrations resulted in premature senescence, chlorotic lesions and earlier chlorophyll breakdown.

SOD activity measured from total leaf extracts showed significant ozone-induced changes after 4 d of fumigation at 100 ppb in both varieties (Fig. 3). The trend of the changes was, however, different in the two varieties; an increase in activity of about 35% was detected for cv. Jinpumbyeo, though there is a slight decrease in activity at early time of fumigation, while a 24% increase was measured in cv. Chucheongbyeo leaves. Moreover a generally higher activity in normal growth condition was registered in cv. Jinpumbyeo. CAT activity in either variety showed similarly throughout ozone fumigation (Fig. 3). No ozone-induced changes were detected in CAT activity until 4 d of ozone fumigation. Subsequent ozone fumigation rapidly elevated the activity of this enzyme to 1.9- (cv. Jinpumbyeo) and 1.7-fold (cv. Chucheongbyeo). POD activity proved to be enhanced by ozone fumigation in both varieties, even if the increase was more pronounced in cv. Jinpumbyeo (60%) than in cv. Chucheongbyeo (31%). This enzyme quickly responded in ozone-treated leaves compared with SOD and CAT activities. Many recent studies have aimed at comparing the antioxidant responses for species or cultivars with different sensitivity to the pollutant (Karlsson *et al.*, 1995; Ranieri *et al.*, 1996). Therefore, in order to understand the differences on the biochemical behavior of the two different rice varieties, we have analyzed their enzymatic antioxidant system. The comparison proved to be in favor of cv. Jinpumbyeo: this variety showed in fact higher levels of the antioxidants

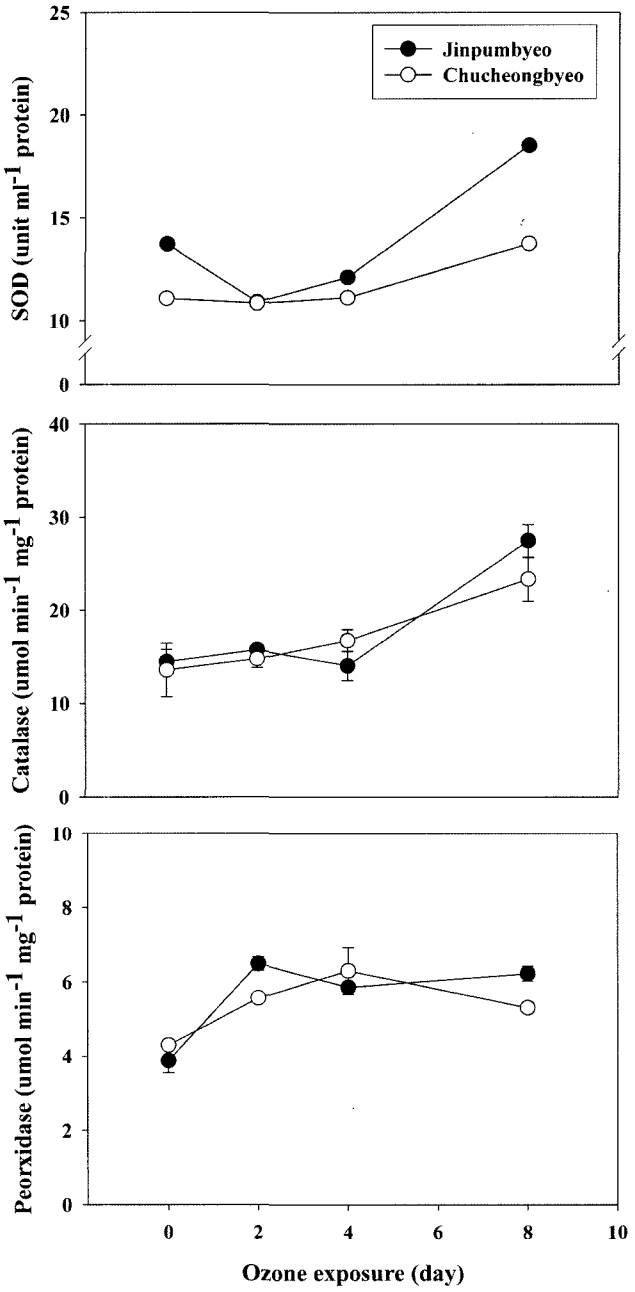

Fig. 3. Antioxidant enzyme activities, SOD, CAT and POD, in ozone treated (100 ± 10 ppb, 4 h) leaves of cv. Jinpumbyeo and cv. Chucheongbyeo for 8 days. Symbols represent mean ± S.D. of three measurements. The statistical *p* values, SOD, CAT and POD, of the variety effect are 0.0001(***), 0.5648(ns) and 0.2860(ns), respectively.

Table 1. Effect of ozone fumigation (O_3) on the concentrations of soluble sugar and starch of leaf blade, leaf sheath and young leaf in both rice varieties

Variety	Treatment (100 ppb d^{-1})	Soluble sugar (mg g^{-1}FW)			Starch (mg g^{-1}FW)		
		Leaf blade	Leaf sheath	Young leaf	Leaf blade	Leaf sheath	Young leaf
Jinpumbyeo	0 d	16.8 ± 0.4	19.6 ± 0.1	12.9 ± 0.4	27.4 ± 1.1	32.5 ± 1.7	41.8 ± 0.7
	2 d	14.1 ± 0.3	14.1 ± 0.8	10.6 ± 0.2	24.0 ± 1.2	30.1 ± 1.8	32.3 ± 1.3
	4 d	13.7 ± 0.3	7.3 ± 0.6	10.2 ± 0.3	16.0 ± 2.0	32.7 ± 1.1	32.4 ± 1.7
	8 d	8.9 ± 0.4	6.6 ± 0.3	10.7 ± 0.4	17.8 ± 0.8	30.6 ± 1.1	41.1 ± 1.3
Chucheongbyeo	0 d	18.8 ± 0.3	20.8 ± 0.8	16.9 ± 0.1	30.7 ± 1.3	30.4 ± 0.8	41.7 ± 4.0
	2 d	14.4 ± 0.1	15.7 ± 0.8	11.3 ± 1.3	23.9 ± 3.7	32.0 ± 0.8	40.4 ± 1.8
	4 d	11.8 ± 0.5	8.1 ± 0.3	10.6 ± 0.4	20.0 ± 1.8	30.5 ± 1.0	38.5 ± 1.6
	8 d	11.0 ± 0.4	8.9 ± 0.3	7.6 ± 0.5	12.0 ± 1.0	29.9 ± 1.7	39.8 ± 1.8
Variety		*	**	ns	ns	ns	ns
Ozone		***	***	***	***	ns	*
Var. * Ozone		***	ns	***	ns	ns	ns

Values are means ± S.D.(n=3); *p<0.05, **p<0.01, ***p<0.001; n.s., not significant.

which could confer better protection against the excessive production of ROS. Ozone increased SOD and APX activities, although these mechanisms do not seem to be completely sufficient to protect plants against oxidative stress (Calatayud *et al.*, 2003). According to the result of Van Camp *et al* (1998) and Pitcher & Zilinskas (1996), SOD played an important role in limiting or preventing lesion development.

Ozone stress significantly reduced soluble sugar and starch contents of all plant parts (Table 1). In cv. Jimpumbyeo, the soluble sugars of leaf blade, leaf sheath and young leaf decreased in 47, 64 and 11%, respectively, whereas those in cv. Chucheongbyeo showed a decrease in 41, 57 and 55%. As a consequence, comparing two varieties, a great difference on the biosynthesis and mobilization of soluble sugars was observed in young leaf, though there is a similar trend in the decrease of soluble sugars of both varieties. Ozone made a less influence on starch than soluble sugars (Table 1). The decrease in starch contents in ozone-induced leaves intensively occurred in leaf blade, and those of leaf sheath and young leaf in both varieties remained almost unchanged compared with the control. The fixation and assimilation of carbon into plant damaged by ROS were inhibited. Photosynthetic apparatus may be a primary target of ozone attack. Leaf chlorophyll concentration, carboxylation efficiency, photosystem II activity, and both the amount and activity of Rubisco are known to be reduced by ozone (Farage *et al.*, 1991; Pell *et al.*, 1994; Pell & Pearson, 1983). As stated above, the lower nitrogen concentration in leaves of cv. Chucheongbyeo (Fig. 2) may indicate impairment due to carbon limitation, because much energy is required for the assimilation of nitrate into protein (Hikosaka & Terashima, 1995). However, there are the controversial data. Ozone increased foliar concentrations of starch and fructans in wheat (Barnes *et al.*, 1995), and soluble sugar contents increased with ozone (Fialho & Bucker, 1996), while starch was unaffected. In other cases, sugar and often starch contents declined or were unchanged by ozone (Miller *et al.*, 1995; Booker, 2000).

In conclusion, ozone adversely affected a lot of biochemical metabolism, though there are slight differences between varieties depending upon ozone sensitivity. Ozone caused the malfunction of stomata due to a decrease in potassium, disturbed carbon fixation and carbohydrate biosynthesis, and besides stimulated high activity of antioxidant enzymes.

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