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EFFECTS OF 24-EPIBRASSINOLIDE ON SALINITY STRESS INDUCED INHIBITION OF SEED GERMINATION, SEEDLING GROWTH AND LEAF ANATOMY OF BARLEY

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Abstract: Amelioration of the inhibitory effect of salt stress (0.0, 0.30, 0.35 molal) on seed germination, seedling growth and leaf anatomy of barley (*Hordeum vulgare* L. cv. Bülbül 89) by infusing of 3 μM 24-epibrassinolide (EBR) to the seeds presowing for 24 hours was investigated. EBR pretreatment was found to be successful in ameliorating of the inhibitory effects of salt stress on final germination percentage, radicle elongation and fresh weight, while it observed to be unsuccessful on final coleoptile percentage and elongation. Besides, EBR pretreatment was mostly determined to have a succesful performance in ameliorating of the inhibitory effects of 0.30 and particularly 0.35 m salinity on leaf anatomy of barley seedlings.

Key words: Barley, brassinosteroids, leaf anatomy, salt stress, seed germination, seedling growth

ARPANIN TOHUM ÇİMLENMESİ, FİDE BÜYÜMESİ VE YAPRAK ANATOMİSİNİN TUZ STRESİ TEŞVİKLİ İNHİBİSYONU ÜZERİNE 24-EPİBRASSİNOLİT'İN ETKİLERİ

Özet: Arpanın (*Hordeum vulgare* L. var. Bülbül 89) tohum çimlenmesi, fide büyümesi ve yaprak anatomisi üzerindeki tuz stresinin (0.0, 0.30, 0.35 molal) engelleyici etkisinin 3 μM 24-epibrassinolit'in (EBR) ekim öncesi tohumlara 24 saat infüzyonu vasıtası ile iyileştirilmesi araştırılmıştır. EBR ön muamelesi nihai çimlenme yüzdesi, radikula uzaması ve taze ağırlık üzerinde tuz stresinin engelleyici etkisini iyileştirmede başarılı olurken, nihai koleoptil yüzdesi ve uzaması üzerinde başarısız olduğu gözlenmiştir. Ayrıca EBR ön muamelesinin arpa fidelerinin yaprak anatomisi üzerinde 0.30 ve özellikle de 0.35 m tuzluluğun engelleyici etkisini iyileştirmede çoğunlukla başarılı bir performansa sahip olduğu tespit edilmiştir.

Anahtar kelimeler: Arpa, brassinosteroitler, yaprak anatomisi, tuz stresi, tohum çimlenmesi, fide büyümesi

INTRODUCTION

Brassinosteroids are steroids that occur in many plant species with common biological activities, suggesting that they are a new group of plant growth hormones (YOKOTA &

TAKAHASHI 1985). Brassinosteroids were first isolated and characterized from the pollen of rape plant, *Brassica napus* L. (MITCHELL et al. 1970). Brassinosteroids have been found to be present in all plants tested so far (9-monocots, 28-dicots, 5-gymnosperm, one pteridophyte and one alga), and based on this, SASSE (1997) suggested that these compounds are probably ubiquitous in the plant kingdom. Brassinosteroids are plant hormones with pleiotropic effects (SASSE 1997), as they influence diverse physiological processes such as seedling growth, seed germination, rhizogenesis, senescense and leaf abscission. One of the most interesting influences of brassinosteroids is their ability to confer resistance to plants against various abiotic stresses. Brassinosteroid-treated tomato and rice plants grew better than control plants under low temperature conditions (KAMURO & TAKATSUTO 1991). Brassinosteroids were shown to increase the tolerance to low and high temperature stress in brome grass (WILEN et al. 1995). A significant influence of brassinosteroids on the recovery of growth by maize and cucumber seedlings after chilling has been demonstrated (HE et al. 1991, KATSUMI 1991). They also confer some resistance to drought stress as reported in the case of sugar beet (SCHILLING et al. 1991) and wheat (SAIRAM 1994). Therefore, brassinosteroids have been reported to alleviation of salinity stress on seed germination and seedling growth (SASSE et al. 1995, ANURADHA & RAO 2001). However, till now, no data have been recorded about effects of brassinosteroids on the leaf anatomy of plants grown under salinity conditions.

In the study reported here, the influence of EBR on seed germination, seedling growth and leaf anatomy of barley subjected to salinity stress was investigated.

MATERIAL AND METHODS

Seed Germination

Experiments have been performed at constant temperature (20°C), in an incubator and continuous dark. Firstly sufficient number of plump, similar sized barley seeds were kept in constant volumes of distilled water (control, C) and EBR (3µM) for 24 hours at room temperature. At the end of this pretreatment session, the solutions were immediately filtered and the seeds were vacuum-dried (BRAUN & KHAN 1976). The seeds for each application were put onto two layers of filter papers in 10 cm petri dishes with 7 ml of distilled water or different salt concentrations (0.0, 0.30 and 0.35 molal,m). These salt levels preventing seed germination of barley in a great extent and the concentration of EBR used were determined in the result of a preliminary study. Dishes were then transferred to incubators for germination. Seeds were accepted as germinated when radicle elongation reached 10 mm (UNGAR 1974). At the end of 7^{th} day, following the estimation of final germination percentage, final coleoptile percentages, radicle and coleoptile lengths were determined. Furthermore, with special replicas for the experiments fresh weights of the seedlings were taken. Each treatment was repeated 4 times.

Growth Conditions of Seedlings from Seeds and Anatomical Observations

Germinated seeds for 7 days at 20°C were transferred afterwards to pots with perlit and

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different Hoagland+salt concentrations (0.0, 0.30 and 0.35 m) for 20 days. Growth conditions were set as: temperature 25±2°C, relative humidity 60±% 5, photoperiod 12-hours, light intensity 160 mol $m^{-2}s^{1}$ PAR (white fluorescent lamps). Anatomical sections were provided by microtome from secondary leaves of 20 day-old seedlings.

Anatomical characters determined were performed 10 times with 3 replicas. After stomata number per unit area and epidermal cell number were determined, stomata index was calculated according to MEIDNER & MANSFIELD (1968). Statistical analyses were done using SPSS program and Duncan's multiple range test.

RESULTS

Effects of EBR on the Seed Germination and Seedling Growth under Saline Conditions

In barley seeds, C group and EBR pretreated ones under normal conditions showed similar results in germination and coleoptile percentages. Radicle elongation and fresh weight of the seedlings were suppressed by EBR pretreatment according to C, but coleoptile elongation was promoted (Table 1).

In parallel with concentration rise, the negative effects of salt have increased on all the seedling growth parameters (final coleoptile percentage, radicle and coleoptile elongation, fresh weight of seedlings). For instance, germination percentage of C seeds was 100 % in distilled water, which turned 20 % at 0.30 m and 0.0 % at 0.35 m salinity. In other words, salt has greatly inhibited germination percentage (80 % at 0.30 m and 100 % at 0.35 m), but EBR pretreatment alleviated in a great extent the negative effects of the salt. Despite the positive effects radicle elongation and fresh weight in a similar way, EBR pretreatment could have no effects on coleoptile percentage and elongation (Table 1).

Table 1. Various growth parameters of the seedlings from barley seeds germinated in the saline media for 7 day

NaCl (m)	Growth regulator	Growth parameters				
		Germination percentage	Coleoptile percentage	Radicle length (mm)	Coleoptile length (mm)	Fresh weight (mg/seedling)
0.0	**C**	*100±0.0 ^{e}	100±0.0 ^{b}	78.6±0.9 ^{e}	81.4±0.6 ^{b}	284.8±1.4 ^{f}
	EBR	100±0.0 ^{e}	100±0.0 ^{b}	61.4±1.1 ^{d}	105.0±1.4 ^{c}	267.5±0.5 ^{e}
0.30	**C**	20±0.0 ^{b}	0.0±0.0 ^{a}	10.8±0.6 ^{b}	0.0±0.0 ^{a}	83.0±0.6 ^{b}
	EBR	60±0.0 ^{d}	0.0±0.0 ^{a}	11.7±0.4 ^{c}	0.0±0.0 ^{a}	96.4±0.6 ^{d}
0.35	**C**	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}	0.0±0.0 ^{a}
	EBR	28±0.0 ^{c}	0.0±0.0 ^{a}	10.5±0.3 ^{b}	0.0±0.0 ^{a}	94.2±0.8 ^{c}

*Shows values with insignificant difference ($P<0.05$) for each column shown with same letters.

Effects of EBR on the Leaf Anatomy under Saline Conditions

EBR pretreatment increased stomata number, epidermis cell number, stomata index, epidermis cell width and leaf thickness in the leaves of barley seedlings grown under normal conditions, while it reduced stomata width and distance between vascular bundles. Although EBR pretreatment increased stomata length on upper surface of the leaves according to C, it decreased this parameter on lower surface (Table 2, Figure 1-3).

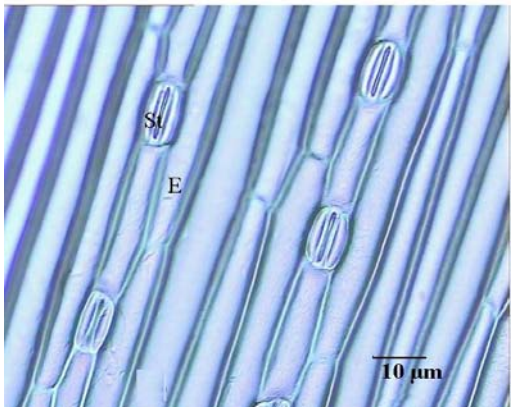

Figure 1. Lower leaf superficial section from C group barley seedlings grown in distilled water St, stomata; E, epidermis

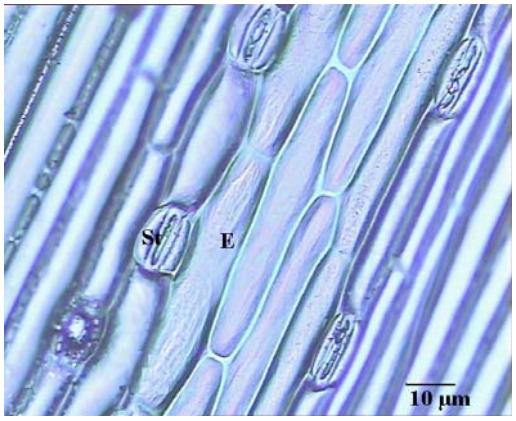

Figure 2. Lower leaf superficial section from EBR pretreated barley seedlings grown in distilled water

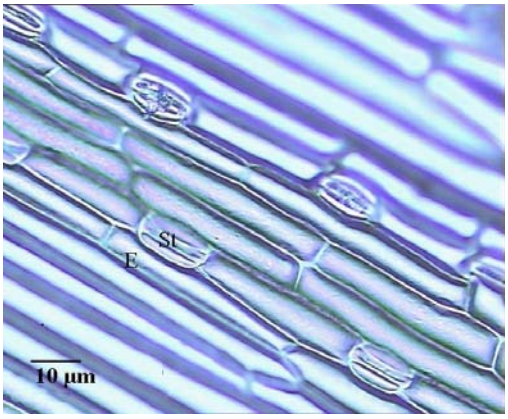

Figure 3. Upper leaf superficial section from EBR pretreated barley seedlings grown in distilled water

Although 0.30 m salinity increased stomata number (lower), epidermis cell number, stomata index (lower), epidermis cell width (upper) and leaf thickness in the leaves of C seedlings, it decreased stomata number (upper), width, length and stomata index (upper). However, it had no effects on epidermis cell width (lower) and distance between vascular bundles. Because C group seeds could not germinate at 0.35 m salinity, leaf anatomy parameters could not be obtained (Table 2).

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On the other hand, EBR pretreatment has increased stomata number, epidermis cell number, epidermis cell width, leaf thickness and distance between vascular bundles in the leaves of barley seedlings growth at 0.30 m salinity according to C. It reduced stomata width, length and stomata index on lower surface of the leaves, while it increased these parameters on upper surface (Figure 4-6). Moreover, EBR pretreated seedlings accomplished growth at high salinity such as 0.35 m and they were determined to have healty leaf anatomy parameters. In other words, EBR pretreatment alleviated the inhibitory effect of 0.35 m salinity on all the leaf anatomy parameters. For example, stomata number, epidermis cell number, epidermis cell width, leaf thickness and distance between vascular bundles values of EBR pretreated seedlings were higher than C group ones grown under normal conditions (Table 2, Figure 7).

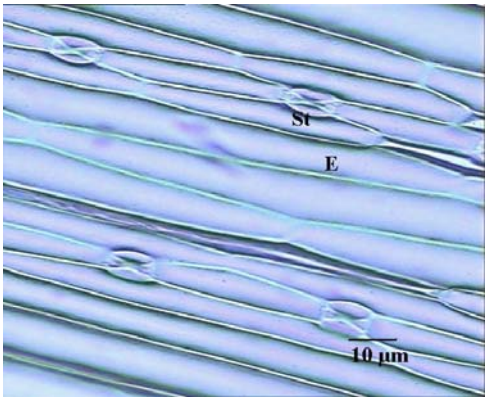

Figure 4. Lower leaf superficial section from EBR pretreated barley seedlings grown at 0.30 m NaCl

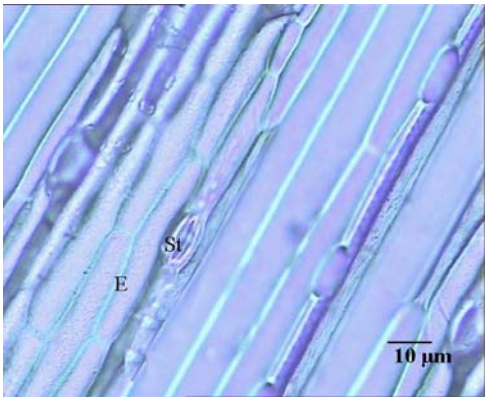

Figure 5. Upper leaf superficial section from EBR pretreated barley seedlings grown at 0.30 m NaCl

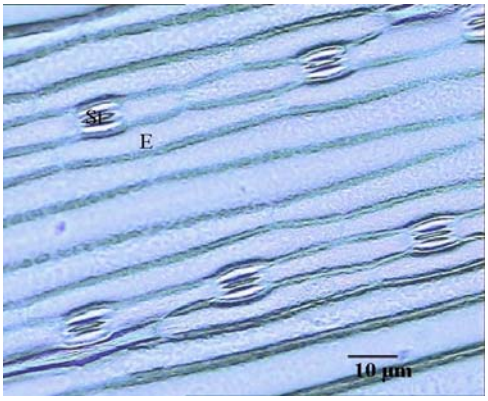

Figure 6. Leaf cross section from EBR pretreated barley seedlings grown at 0.30 m NaCl

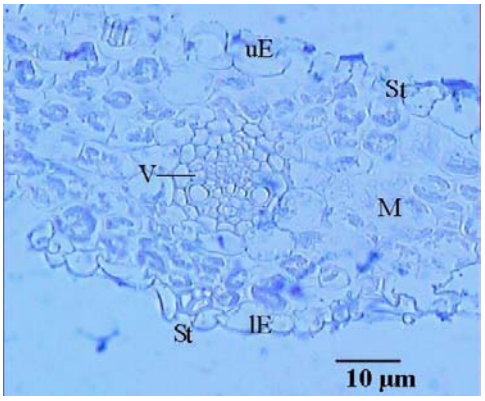

Figure 7. Upper leaf superficial section from EBR pretreated barley seedlings grown at 0.35 m NaCl, uE, upper epidermis; IE, lower epidermis; St, stomata; M, mesophyll cells; V, vascular bundle

Table 2. Leaf anatomy parameters of barley seedlings grown in various concentrations of NaCI at 25ºC after EBR pretreatment by 20th day

NaCl (m)	Growth regulator	Stomata number		Epidermis cell number		Stomata width (µm)		Stomata length (µm)		Stomata index		Epidermis cell width (µm)		Leaf thickness (µm)	Distance between vascular bundles (µm)
		Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower		
0.0	**C**	*3.3±1.1^{b}	3.7±1.1^{a}	6.4±0.8^{a}	10.8±0.7^{a}	8.2±1.1^{d}	10.1±0.8^{d}	14.4±1.6^{c}	15.8±0.8^{c}	33.8	25.7	5.6±0.6^{a}	5.4±0.6^{a}	44.0±2.1^{a}	78.3±1.1^{b}
	EBR	4.3±0.8^{c}	7.1±1.1^{d}	8.0±1.2^{b}	11.7±1.6^{b}	7.9±1.3^{c}	8.4±1.2^{c}	15.1±1.8^{d}	13.1±0.9^{a}	35.2	37.6	7.0±0.9^{c}	6.6±0.9^{c}	54.6±1.7^{b}	60.0±1.5^{a}
0.30	**C**	2.4±0.9^{a}	5.6±1.1^{b}	10.3±1.2^{c}	11.8±1.3^{b}	7.3±1.4^{b}	7.9±1.1^{b}	13.2±1.7^{a}	14.5±1.1^{b}	14.4	32.1	6.3±1.4^{b}	5.6±0.9^{a}	63.3±2.4^{c}	80.5±1.3^{b}
	EBR	4.8±0.8^{d}	6.3±1.4^{c}	14.2±1.3^{d}	13.6±2.3^{d}	9.8±0.9^{e}	7.1±0.6^{a}	14.1±1.6^{b}	12.5±1.5^{a}	31.6	31.8	7.8±1.1^{d}	10.9±1.1^{d}	70.2±1.5^{e}	87.4±2.8^{c}
0.35	**C**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	EBR	4.5±0.9^{d}	5.4±0.9^{b}	14.3±0.7^{d}	12.5±1.5^{c}	6.9±1.3^{a}	6.5±0.6^{a}	12.2±0.7^{a}	12.9±1.2^{a}	24.1	30.1	7.0±0.8^{c}	6.4±0.6^{b}	67.6±2.1^{d}	86.0±1.6^{c}

* Shows values with insignificant difference ($P<0.05$) for each column shown with same letters

- The C seeds could not germinate in the marked concentrations

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DISCUSSION

Generally unless the stress conditions are present, there is no need to add exogenously any hormones in germination process. But roles of brassinosteroids in normal conditions are not known well. Thus we also wanted to test effects of this growth regulator on seed germination, seedling growth and leaf anatomy under normal conditions. As seen in Table 1, for the germination and coleoptile percentage, hormone treatment resulted in similar results with those of the C group in the medium of distilled water such as many studies showing positive effect under normal conditions (JONES-HELD et al. 1996, LEUBNER-METZGER 2001, CAVUSOGLU 2006). Our results showed that EBR pretreatment reduced radicle elongation and fresh weight of the seedlings, while promoted coleoptile elongation (Table 1). Effects of brassinosteroids on radicle elongation under normal conditions are still unclear. Despite studies mentioning negative effects of brassinosteroids on radicle elongation of Arabidopsis (CLOUSE et al. 1993) and soybean (HUNTER 2001) seedlings, other studies report promoting effect on wheat (SASSE 1994) and chickpea (SINGH et al. 1993) seedlings. As a result EBR affect radicle elongation in different ways depending on plant taxon, applied concentrations and pretreatment styles. Similarly promoting effects of brassinosteroids on stem elongation of Arabidopsis, wheat and tomato have been previously reported (SASSE 1985, STEBER & MCCOURT 2001). However, neutral or negative effects on this character have also been recorded (ONO et al. 2000, HUNTER 2001). Moreover, OZDEMIR et al. (2004) reported decreasing effect on root fresh weight and increasing effect of brassinosteroid on stem fresh weight of rice seedlings.

The inhibitory effects of salt stress on germination process are universally accepted (GHOULAM & FORES 2001, GULZAR & KHAN 2002). Increasing salinity levels showed inhibitory effect on the seedling growth parameters such as germination of barley seeds (Table 1). Preventive effects of salt stress on coleoptile percentage (CAVUSOGLU 2006), root and stem elongation (DASH & PANDA 2001), fresh weight and water content (EL-MASHAD & KAMEL 2001, CAVUSOGLU 2006) have been shown previously. Our results showing decrease in fresh weight and water content in saline medium can be explained with inability of water uptake due to high osmotic pressure of the medium (BOHNERT et al. 1995, AL-KARAKI 2001). Salt induced-inhibition of root and stem elongation must relate with together with osmotic effect, inhibition of DNA, RNA and protein synthesis (ANURADHA & RAO 2001) or decreasing in the amount of endogen growth stimulating hormones (WALKER & DUMBROFF 1981) and increasing of inhibitory hormones due to salt stress (MIZRAHI et al. 1971).

On the other hand, EBR pretreatment has clearly overcame the inhibitory effects of salt stress on seed germination, radicle elongation and fresh weight. However, it had no effects in ameliorating on coleoptile percentage and elongation (Table1). Brassinosteroids have been reported to increase seed germination (SASSE et al. 1995, OZDEMIR et al. 2004), radicle elongation (CAVUSOGLU 2006) and fresh weight (ANURADHA & RAO 2003), while to inhibit coleoptile percentage (CAVUSOGLU 2006) and stem elongation (OZDEMIR et al. 2004). EBR may be effective on the growth and development by increasing nucleic acid and protein synthesis (CLOUSE et

al. 1993, BAJGUZ 2000) or by reducing endogenous amounts of inhibiting hormones (EUN et al. 1989).

Our results showed that EBR pretreatment reduced stomata width, length (lower) and distance between vascular bundles in the leaves of barley seedlings grown under normal conditions, while it increased the other leaf anatomy parameters (Table 2). However, Effects of brassinosteroids on the leaf anatomy of seedlings grown under normal conditions are little known. Promoting effects of brassinosteroids on stomata number, epidermis cell number, epidermis cell width and leaf thickness have been previously reported (HU et al. 2000, ONO et al. 2000, ARTECA & ARTECA 2001), in conformity with our results.

The anatomical structure of *H.vulgare* was greatly affected by the NaCI concentration of the growth medium. 0.30 m salinity reduced stoma number (upper), width, length and stomata index (upper), while it increased stomata number (lower), epidermis cell number, stomata index (lower), epidermis cell width (upper) and leaf thickness of barley leaves. It had no effects on epidermis cell width (lower) and distance between vascular bundles. Because C group seeds could not germinate at 0.35 m salinity, leaf anatomy parameters could not be obtained (Table 2).

Preventive effects of salt stress on stomata number (KEMP & CUNNINGHAM 1981, FLOWERS et al. 1986, HWANG & CHEN 1995), stomata index (BRAY & REID 2002), epidermis cell number and width (CURTIS & LAUCHLI 1987) and leaf thickness (SHENNAN et al. 1987, RAWSON et al. 1988, YEO et al. 1991, HU & SCHMIDHALTER 2001) have been previously reported. However, promoting effects of salt stress on stomata number (CURTIS & LAUCHLI 1987), leaf thickness (HWANG & CHEN 1995) and epidermis cell number (BRAY & REID 2002) have also been recorded. ROBINSON et al. (1983) determined that plants exposed to salinity stress had higher concentrations of Na^+ and CI^-, and lower concentration of K^+, in new leaf tissue than unstressed plants. Besides, they reported that photosynthesis was also reduced in salt stressed plants, and this reduction was due to limitations on stomatal conductance. Na^+ and CI^- affect cell division and duration of cell elongation. Salt stress may inhibit cell division by causing the accumulation of ABA. The increase of ABA concentration in the growth zone of the maize leaves by salt stress has been observed by CRAMER & QUARRIE (2002).

Our results showed that EBR pretreatment has increased stomata number, epidermis cell number, width, leaf thickness and distance between vascular bundles in the leaves of barley seedlings grown 0.30 m salinity. But it reduced stomata width, length and stomata index on lower surfaces of the leaves, while it increased these parameters on upper surfaces. Furthermore, EBR pretreatment had clearly overcame the inhibitory effects of 0.35 m salinity on the leaf anatomy of barley seedlings (Table 2). However, till now, we have not recorded any data about effects of brassinosteroids on the leaf anatomy of plants grown under salinity conditions. To clarify the effect mechanism(s) dealt chemical further research, fine and detailed, is needed and we believe that our study will contribute to future studies.

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