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Full Length Research Paper

In vitro salt stress induced production of gymnemic acid in callus cultures of *Gymnema sylvestre* R.Br.

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***Gymnema sylvestre*, a well known antidiabetic plant, was cultured *in vitro* for callus induction using leaf explant. Gymnemic acid content enhancement under the influence of different plant hormones like 2,4-D, BA with or without NaCl supplementation to the MS medium is presented in this investigation. The highest efficiency of callus formation was observed in the medium containing different concentrations of 2,4-D. The gymnemic acid content increased with increasing concentration of 2,4-D along with NaCl.**

Key words: *In vitro, Gymnema sylvestre*, callus culture, gymnemic acid, salt stress.

INTRODUCTION

The use of *Gymnema sylvestre* R.Br. (Asclepiadaceae) commonly known as “Gurmar” (destroyer of sugar) or “Periploca of the woods” is well-known to the Indian people since ancient days (“Meshashring”) as a source of antidiabetic drugs. In recent years, it became one of the better known names in the world of herbal medicine. It is a rich source of many bioactive compounds such as gymnemic acid (GA-I-X) quercitol, lupeol, β-amyrin, stigmasterol, gymnemin, gymnemagenin, gurmarin, etc. which are mainly effective in lowering of blood sugar.

The normal propagation method of *G. sylvestre* requires a lot of time (about 6 - 8 months) to grow a developed plantlet from seed. In addition, it requires sufficient field, constant manuring and a constant observation which in turn requires a lot of manpower. Apart from this, getting seeds from the plant is difficult and moreover the chance of getting a disease/contamination free plant is less. On the contrary, a standardized method of micropropagation of *G. sylvestre* can provide a greater percentage of yields of pathogen free plant in a shorter time and in a smaller place. Also, in order to obtain those active compounds, one need not regenerate several complete plants (Jha and Ghosh, 2005). Extracts from pathogen free calli if generated *in vitro* will prove beneficial.

The present study describes the procedure for the callus induction and culture of *G. sylvestre* following standard plant tissue culture protocol using different meristematically active plant parts, growth regulators and studying their effect on callus induction and gymnemic acid production.

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Abbreviations: 2, 4-D, 2, 4-Dichlorophenoxyacetic acid; **BA,** N6-benzylaminopurine; **PGR,** plant growth regulators; **MS,** Murashige and Skoog; **Kn,** kinetin; **HPTLC,** high performance thin layer chromatography.

MATERIALS AND METHODS

Young leaves of *G. sylvestre* were collected from the plants grown in the botanical garden of the department. The leaves were surface sterilized first by washing with 70% alcohol followed by 0.1% mercuric chloride for 2 - 3 min. The leaves were then thoroughly rinsed with double distilled sterilized water 2 - 3 times and directly inoculated on nutrient medium under sterilized conditions of laminar flow bench. Standard techniques of media preparation, inoculation and incubation were followed. Throughout, callus was reared on Murashige and Skoog (1962) medium supplemented with various plant growth regu-lators (PGR). Cultures were maintained at 25 ± 2°C in continuous light of 1400 lux intensity. The observations were taken after fixed time intervals.

Extraction procedure of gymnemic acid

Total gymnemic acid was determined by high performance thin layer chromatography (HPTLC) analysis (Hong et al., 1992) as gymnemagenin which was the main pogenin obtained up on hydrolysis

of the mixture of the gymnemic acid in the extract.

Preparation of test solution

About 0.75 g of the sample was weighed accurately and dissolved in 50% ethanol to make 50 ml. To 10 ml of this solution, was added 2 ml of 12.0% KOH and heated on a boiling water bath for 1 h. After cooling, 5.5 ml 4 N HCl was added and heated on a boiling water bath for 1 h. After cooling, 12.0% KOH was added to raise pH up to 8.0 ± 0.5 and 50.0% ethanol was added to make final volume upto 100 ml. The extract was filtered and made upto 100 ml with 50.0% ethanol. It was filtered again and subjected to evaporation and the dry weight was weighed with soxhlet apparatus. Final concentration of 20 mg/ml with HPLC grade ethanol was prepared and 20 µl was loaded on HPT LC.

Preparation of a standard solution

The major bioactive constituents of *G. sylvestre* are a group of oleanane type triterpenoid saponins known as “gymnemic acids”. Non-availability of the different reference standards makes the job more difficult, therefore the estimation of the different gymnemic acids was performed by hydrolyzing the extract first with alkali and then with acid. The gymnemagenin thus obtained was estimated by HPTLC and the total gymnemic acid content was calculated.

High performance thin layer chromatography (HPTLC) operating conditions

Samples prepared as mentioned above were analyzed for gymnemic acid with gymnemagenin and rutin as standard; the condition for analyses were maintained as follows: HPTLC precoated 20 × 20 cm (width × height) Silica Gel MERCK $60F_{254}$ plates were used. Sample was applied at 10 mm position and allowed to run upto 80 mm height in a CAMAG twin trough chamber. A distance of 12 mm was maintained between tracks and the total number of tracks on one plate was not more than 15. Chloroform: methanol: acetic acid (5:1:1) was used as mobile phase. For detection, a tungsten lamp as light source was used.

RESULTS AND DISCUSSION

Callus induction

Callus induction occurred best on 2.0 mg/l 2, 4-D and 0.05 mg/l BA supplemented MS medium, and very poor to no callus developed on BA (0.5 - 2.0 mg/l) and 2,4-D (4.0 mg/l) supplemented sets. 0.5 - 2.0 mg/l 2, 4-D induced brown friable callus within 15 - 20 days (Table 2 and Plate 1), but in combination with BA (0.1 - 2.0mg/l) green and compact callus developed in at least 20 days. These results are in contradiction with Roy et al. (2008), who reported friable green callus on 2,4-D and 2,4-D+ Kn. Interestingly, on 3.0 mg/l 2,4-D compact brown callus in about 20 days was developed in the present investigation, whereas, Roy et al. (2008) reported friable fluorescent green callus in about 25 days using the same explant (leaf). In the present studies BA was preferred over Kn used by Roy et al. (2008). Addition of salt (NaCl) up to 150 mM, to MS + 0.5 - 3.0 mg/l 2,4-D led to anthocyanin formation in the fragile brown callus (Table 1 and Plate 1).

This is in contrast with the report on *Vitis vinifera* callus, where anthocyanin accumulation reduced callus growth under osmotic stress (Do and Cormier, 1990). Probably salt stress does not deactivate the enzymes required for growth upon addition of salt in *Gymnema*. Such high callus growth suggests it to be unfailingly salt tolerant. Gossett et al. (1994) studied two cultivars of cotton and declared one to be salt tolerant on the basis of less reduction in callus growth under high concentration of NaCl (150 mM). Kavi (1989) is also of the view that higher growth under salt concentration as high as 100 - 200 mM is suggestive of the cultivar to be salt tolerant as in rice.

Gymnemic acid content

As noted earlier, higher concentration of 2,4-D with or without NaCl and combination of higher concentration of 2,4-D and BA with NaCl indicate stress tolerance in callus through accumulation of protein, proline, sugars and polypeptides; gymnemic acid content accumulation also supports the same. Thus, the gymnemic acid content increased with increasing concentration of 2,4-D alone and also with NaCl. 1.0 mg/l 2,4-D + 1.0 mg/l BA with NaCl appear to cause lesser stress to the callus than 3.0 mg/l 2,4-D with 0.05 mg/l BA without NaCl. Highest stress could be noted with 3.0 mg/l 2,4-D + 1.0 mg/l BA + 100 mM NaCl. Some secondary peaks related to gymnemic acid also appeared, especially in the absence of salt, which might indicate that salt could deactivate the metabolic pathway leading to synthesis of these unknown compounds. At the moment, it cannot be said that with the fragile information available in the literature, to what are these compounds and what could be their role, why are they found associated with gymnemic acid and do they have any therapeutic activity (Table 3)?

Subhadra et al. (2006) and Gopi and Vatsala (2006) have reported production of gymnemic acid from suspension and callus cultures of *G. sylvestre*. Gopi and Vatsala (2006) claim to procure higher gymnemic acid from callus than the explant, without any supporting data. However, there is no report regarding such increase in gymnemic acid from salt stressed calli under 2,4-D + BA treatment (Figure 1). Besides, additional peaks similar to gymnemic acid standard might be the co-metabolites in the callus cultures which might support the activity of gymnemic acid. The present investigation may be a gateway for molecular pharming of gymnemic acid, when the plant itself is declared vulnerable in the red data book.

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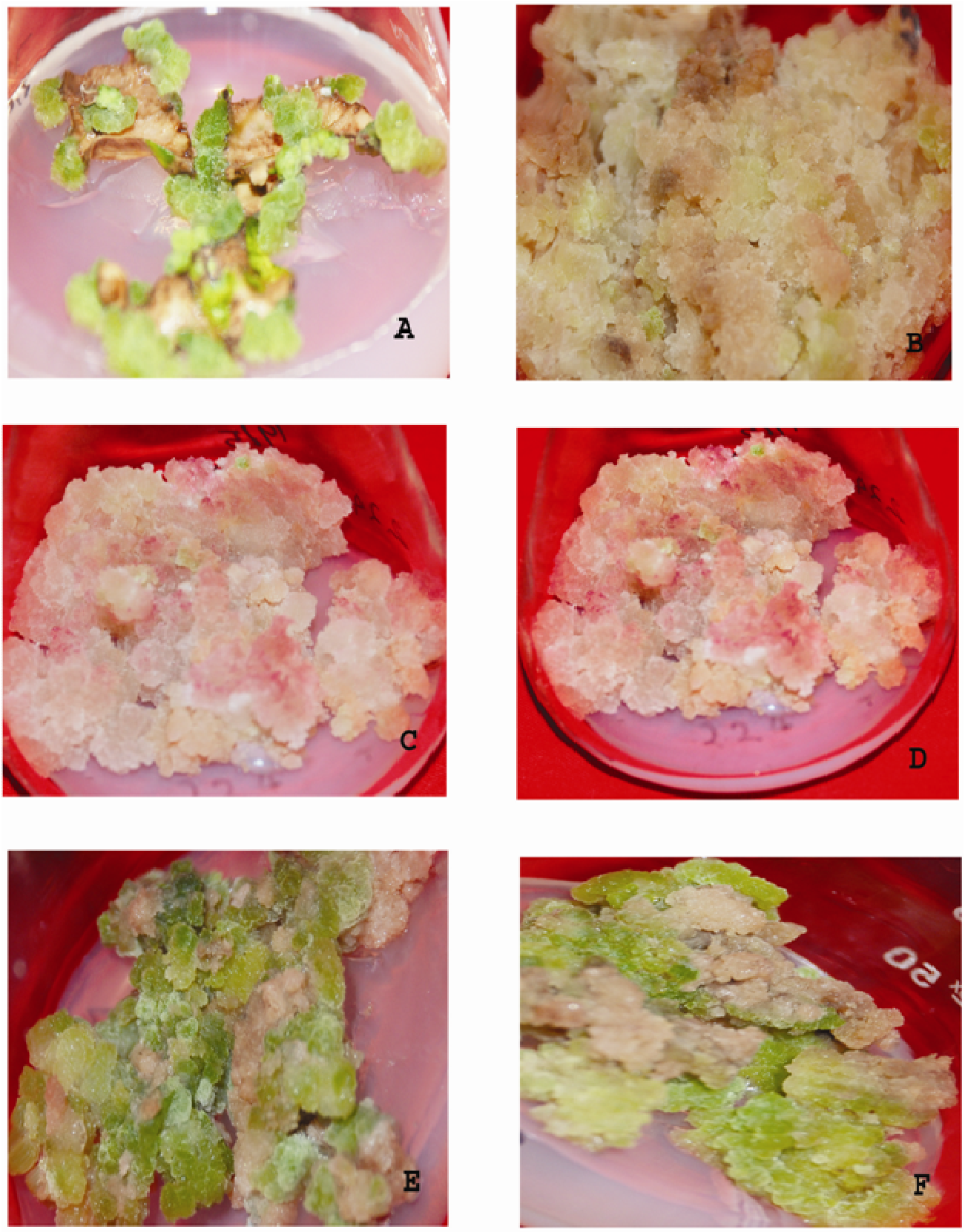

Plate 1. A, Callus induction from leaf explant on MS + 2,4-D (1.0 mg/l) + BA (2.0 mg/l); B, callus on MS + 2,4-D (2.0 mg/l) + BA (0.05 mg/l); C, callus on MS + 2,4-D (2.0 mg/l) + 100 mM NaCl; D, callus on MS + 2,4-D (2.0 mg/l) + 150 mM NaCl; E, callus on MS+2,4-D (2.0 mg/l) + BA (1.0 mg/l); and F, callus on MS + 2,4-D (2.0 mg/l) + BA (2.0 mg/l).

Table 1. Effect of 2,4-D on four week old callus of *G. sylvestre.*

S/N	MS +3% Sucrose + (mg/l) PGRs	Salt (NaCl)	Callus growth	Morphology of callus
1	0.5 2,4-D	-	+++	Fragile and brown
2	0.5 2,4-D	50mM	++++	Fragile and brown
3	0.5 2,4-D	100mM	+++	Fragile and brown
4	0.5 2,4-D	150mM	++	Fragile, brown and anthocyanin formation
5	1.0 2,4-D	-	++++	Fragile and brown
6	1.0 2,4-D	50mM	++++	Fragile and brown
7	1.0 2,4-D	100mM	+++	Fragile, brown and anthocyanin formation
8	1.0 2,4-D	150mM	++	Fragile, brown and anthocyanin formation
9	2.0 2,4-D	-	++++	Fragile and brown
10	2.0 2,4-D	50mM	++++	Fragile, brown and anthocyanin formation
11	2.0 2,4-D	100mM	+++	Fragile, brown and anthocyanin formation
12	2.0 2,4-D	150mM	++	Fragile, brown and anthocyanin formation
13	3.0 2,4-D	-	++++	Fragile and brown
14	3.0 2,4-D	50mM	+++	Fragile and brown
15	3.0 2,4-D	100mM	+++	Fragile, brown and anthocyanin formation
16	3.0 2,4-D	150mM	++	Fragile, brown and anthocyanin formation

++++ Excellent response; +++ good response; ++ medium response.

Table 2. Effect of 2,4-D, BA and NaCl on four week old callus of *G. sylvestre*.

S/N	MS + 3% sucrose + (mg/l) PGRs	SALT	Callus growth	Morphology of callus
1	1.0 2,4D+0.05BA	-	+++	Fragile and light green
2	1.0 2,4D+0.05BA	50mM	++++	Fragile and light green
3	1.0 2,4D+0.05BA	100mM	+++	Fragile, light green and anthocyanin formation
4	1.0 2,4D+0.05BA	150mM	++	Fragile, light green and anthocyanin formation
5	1.0 2,4D+1.0BA	-	+++	Highly compact and dark green
6	1.0 2,4D+1.0BA	50mM	+++	Compact and dark green
7	1.0 2,4D+1.0BA	100mM	++	Compact and dark green
8	1.0 2,4D+1.0BA	150mM	++	Compact and white
9	2.0 2,4D+0.05BA	-	++++	Compact and white
10	2.0 2,4D+0.05BA	50mM	++++	Compact and white
11	2.0 2,4D+0.05BA	100mM	+++	Compact and brown
12	2.0 2,4D+0.05BA	150mM	++	Compact and brown
13	2.0 2,4D+1.0BA	-	+++	Fragile and brown
14	2.0 2,4D+1.0BA	50mM	+++	Highly compact and dark green
15	2.0 2,4D+1.0BA	100mM	++	Compact and brown
16	2.0 2,4D+1.0BA	150mM	++	Compact and brown
17	3.0 2,4D+0.05BA	-	+++	Fragile and light green
18	3.0 2,4D+0.05BA	50mM	+++	Fragile and light green
19	3.0 2,4D+0.05BA	100mM	++	Fragile and light green
20	3.0 2,4D+0.05BA	150mM	++	Fragile and brown
21	3.0 2,4D+1.0BA	-	++	Highly compact and dark green
22	3.0 2,4D+1.0BA	50mM	+++	Highly compact and dark green
23	3.0 2,4D+1.0BA	100mM	++	Highly compact and dark green
24	3.0 2,4D+1.0BA	150mM	+	Highly compact and dark green

Table 3. Estimation of gymnemic acid in different callus samples of *G. sylvestre*.

S/N	MS +3% sucrose + (mg/l) PGRs	Gymnemic acid content % (w/w)	Other peak in comparison with standard % (w/w)
1	1.0 2,4-D	ND	ND
2	1.0 2,4-D+50 mM NaCl	2.1	ND
3	1.0 2,4-D+100 mM NaCl	4.06	ND
4	3.0 2,4-D	4.19	ND
5	3.0 2,4-D+50 mM NaCl	4.31	0.54
6	3.0 2,4-D+100 mM NaCl	4.4	ND
7	1.0 2,4-D+0.05 BA	ND	5.29
8	1.0 2,4-D+1.0 BA	ND	1.97
9	1.0 2,4-D+1.0 BA+50 mM NaCl	3.58	ND
10	1.0 2,4-D+1.0 BA+100 mM NaCl	3.36	ND
11	3.0 2,4-D+0.0 5 BA	5.09	1.19
12	3.0 2,4-D+1.0 BA	4.36	5.62
13	3.0 2,4-D+1.0 BA+50 mM NaCl	4.02	ND
14	3.0 2,4-D+1.0 BA+100 mM NaCl	5.09	ND

*ND = Not detected.

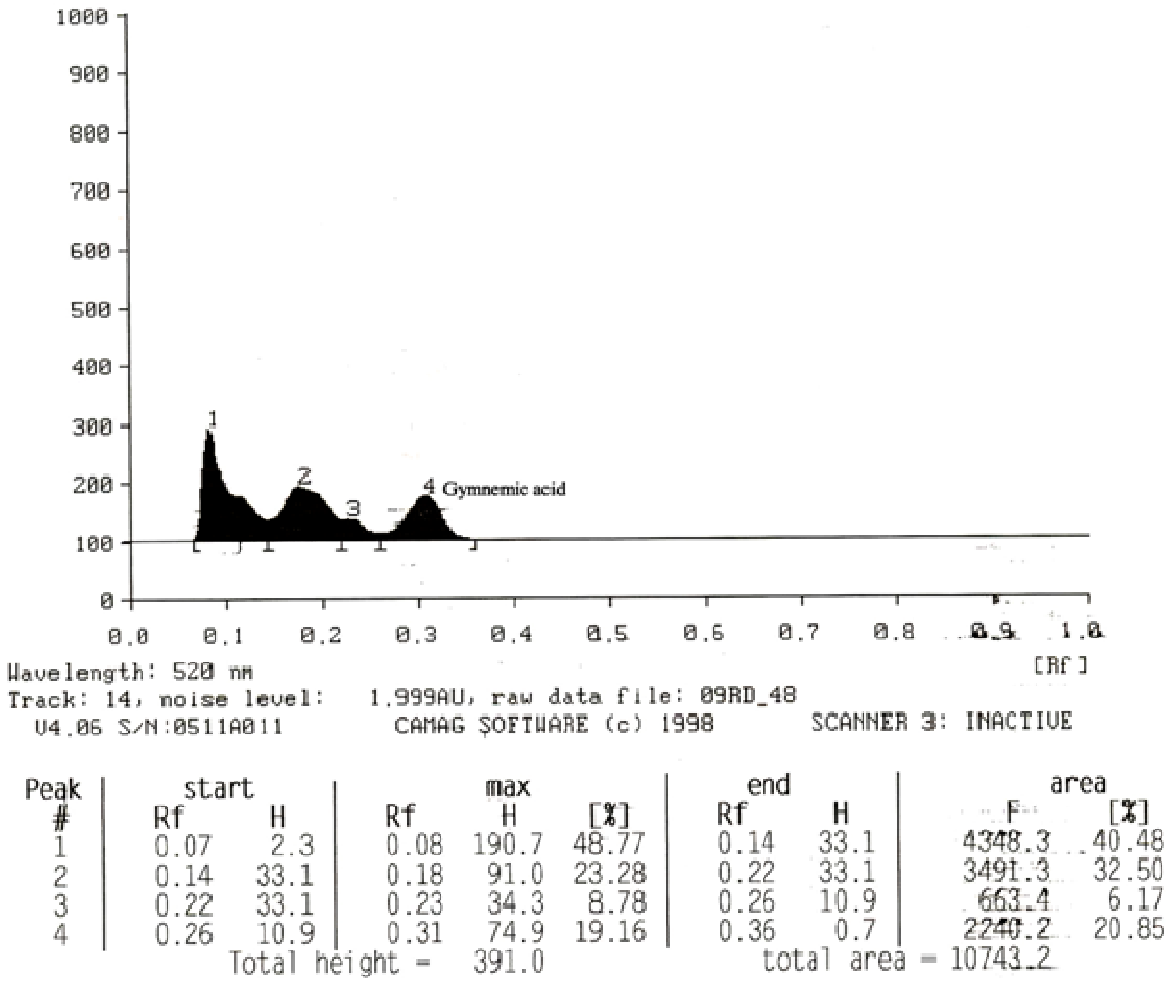

Figure 1. HPTLC profile of *G. sylvestre* 4-week old callus from leaf explants induced on MS + 3.0 mg/l 2,4-D + 1.0 mg/l BA + 100 mM NaCl using gymnemic acid standards.

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