In vitro Plant Regeneration from Hypocotyl and Cotyledon Explants of BARI SARISHA-13 (*Brassica napus L.*)

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ABSTRACT— ***The aim of the present study was to develop an efficient in vitro regeneration system for BARI sarisha-13 (Brassica napus* L.*) where hypocotyls and cotyledonary petioles obtained from in vitro grown seedlings were used as explants for indirect regeneration. Single and combinatorial effects of plant growth regulators (PGRs) and additives were observed. Varying concentrations of hormones was found to change efficiency of callus, shoot and root formation. Increasing 2, 4- dichlorophenoxyacetic acid (2,4-D) from 0.5 mgl^{-1} to above and 6, benzyl amino purine (BAP) concentration from 2 mg^{-1} to 3 mg^{-1} and NAA from 0.1 mg^{-1} to 0.5 mg^{-1} decreased callus and shoot formation, respectively. Roots were obtained when naphtalene acetic acid (NAA) was used. Effects of kinetin, $AgNO_3$, casein hydrolysate (CH), proline, indole 3-butyric acid (IBA), etc were also seen in different stages of regeneration. Percentage and photographs of responsive explants was taken after 24 days from inoculation.***

Keywords—BARI-13, *in vitro* regeneration, PGRs

1. INTRODUCTION

Since the last few decades, oil crops have drawn the attraction of the world agriculture and associated industries because of their economic interest and food value. Refined agronomic practices and plant breeding techniques have opened new doors that have significantly increased oil crop production [1]. Among them, the genus *Brassica* comprises many commercially important vegetable and oilseed crops that are a good source of edible oil and protein - rich product in the world. Mustard (*Brassica* spp.) is such an important annual oil crop that was one of the first domesticated crops of Europe and has subsequently dispersed in the world including Asia.

In Bangladesh mustard oil is an important part of diet that is used for cooking purposes, for salad dressing and to marinate a number of food stuffs before cooking, and also many other health purposes. But the productivity and quality of these crops are affected by various pest and pathogen attacks. More than 10 diseases are reported in Bangladesh rapeseed and mustard. Leaf blight disease, caused by *Alternaria brassicae* [2] is one of the most important diseases. Alarmingly, 30-40% heavy loss in the crop yield has been reported due to this disease [3, 4].

To increase both yield and quality, plant breeders have been spending decades. The conventional breeding techniques vary considerably from simple mass selection to the development of hybrid cultivar. The backcross technique is a very well established one for improving seed quality such as low erucic acid [5]. Conventional oil crop breeding have been performed with outcome of many better cultivars but oil crop breeding is complex than breeding of cereals and legumes due to the requirement of simultaneous manipulation of different traits. For *Brassica*, it is more difficult and takes long time to develop a new variety [6]. Genetic manipulation by *Agrobacterium tumifaciens* has been proved to a successful process and *in vitro* regeneration is an indispensible stage for that. Though Brassicas are usually considered to be unresponsive [7] in tissue culture, many reports of *in vitro* regeneration are now available for different *Brassica* spp. [8, 9, 10, 11, 12]. However, *in vitro* regeneration information on BARI-13 is limited. Hence, the present study was carried out to develop a genotype independent *in vitro* tissue culture system for the local *Brassica* variety, BARI-13.

2. MATERIALS AND METHODS

Materials

Plant materials: BARI Sharisha -13 (*B. napus* L.*)* was used for *in vitro* callogenesis and regeneration. Healthy seeds were collected from Bangladesh Agricultural Research Institute (BARI), Hathazari Substation, Chittagong.

Explants: The explants collected from *in vitro* grown seedlings used for the experiments were hypocotyls and cotyledons with petiole. Hypocotyls were cut into 0.5-1.0 cm pieces and the cotyledons were cut along petioles

Methods

Half strength MS [13] medium was used for seed germination whereas different combinations and concentrations of PGRs (Plant Growth Regulators) and additives were used for callus induction, shooting and rooting. MS media for callogenesis were supplemented with various concentration of 2, 4-D and 30 g l-1 (w/v) sucrose. BAP was also used at a concentration of 0.5 mg l-1 in addition to 2, 4-D. Additives such as proline and CH and media with or without AgNO3 were also used in some combinations. For the development of shoots from the calli, BAP, NAA and Kinetin were used with MS media. For the induction of roots IAA and IBA were used. pH was adjusted at 5.8 for all media. The incubation temperature was maintained at 26±2º C in the culture room and a 16 hour photoperiod was maintained. Regenerated *in vitro* plants were successfully transplanted to soil.

Sterilization of explants and media: Work was performed under the hood of laminar airflow. Collected BARI-13 seeds were immersed in 70% Ethanol for 10 mins. with occasional shaking followed by rinsing with double distilled water. Seeds were then placed in 0.1% HgCl2 solution for another 10 minutes with periodical shaking and washed five to seven times. Seeds were then placed on 70 mm sterile filter paper and left for 10 minutes to remove excess water. All the media and glasswares were sterilized by autoclaving 20 mins. at 121 ºC.

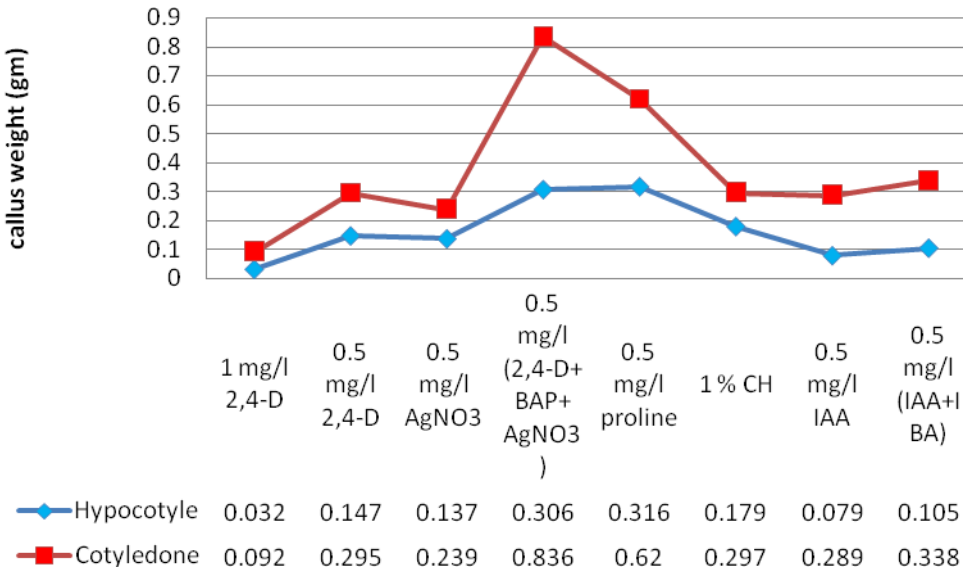

Fig.1. Average weight (gm) of hypocotyl and cotyledonary explants of BARI-13 on different callus induction media, Weight taken after 28 days from inoculation

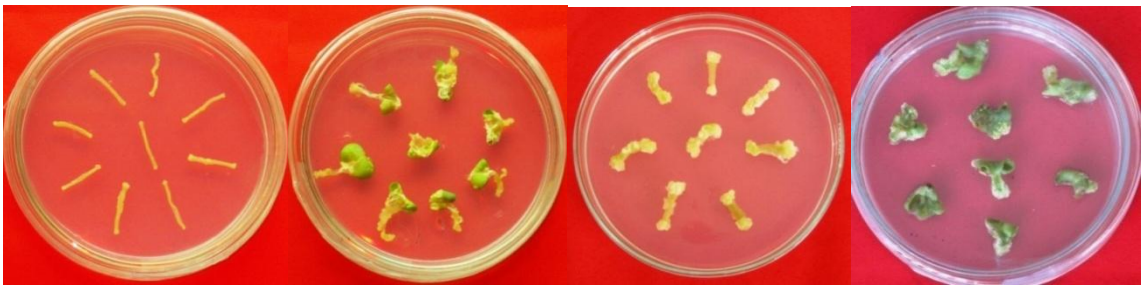

Fig.2. Combinatorial effect of BAP and $AgNO_3$ on callogenesis; initiation of callus from BARI-13 (A) hypocotyls, (B) cotyledons on medium supplemented with only 1.0 mg l^{-1} 2, 4-D but no BAP and $AgNO_3$, and initiation of callus from (C) hypocotyls, (D) cotyledons on medium supplemented with 1.0 mg l^{-1} 2, 4-D, 0.5 mg l^{-1} BAP, and 0.5 mg l^{-1} $AgNO_3$

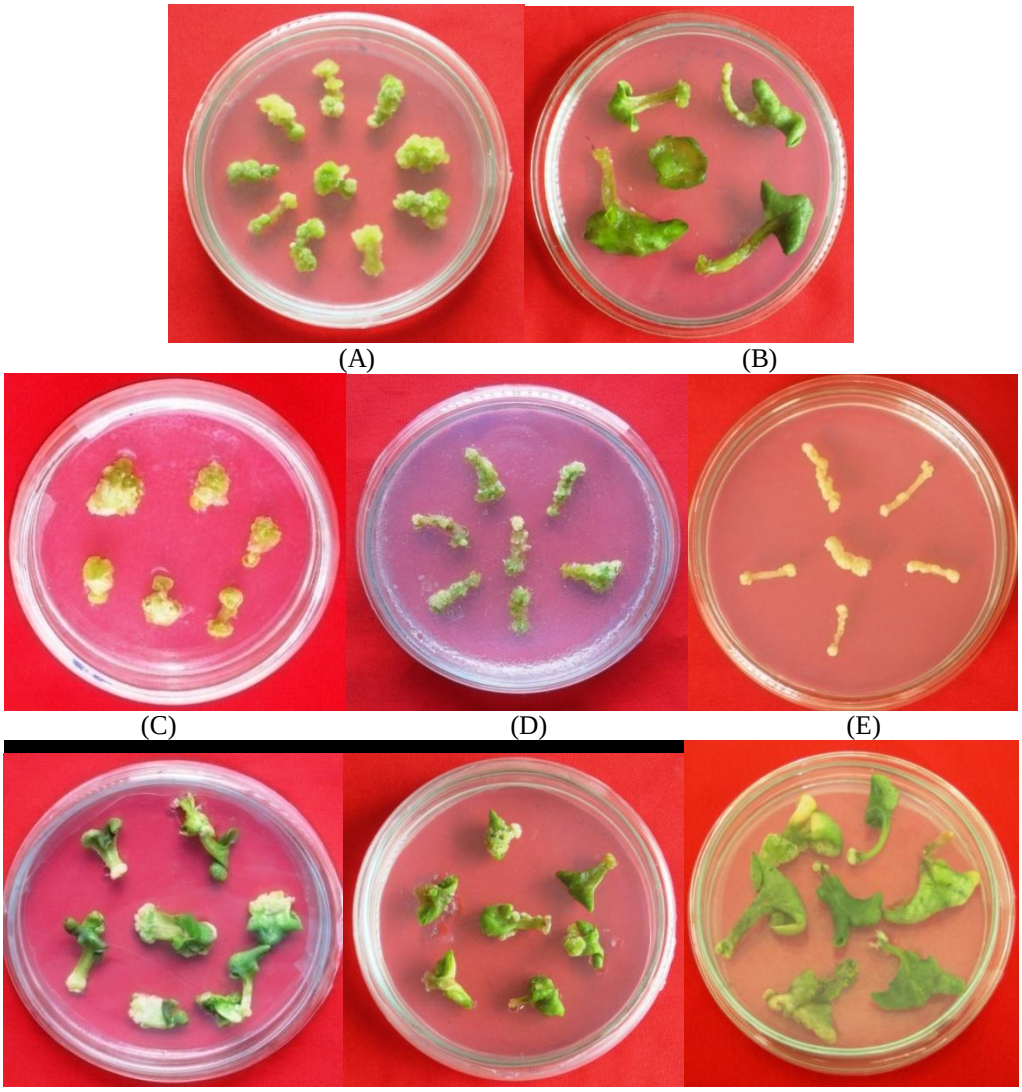

Fig.3. Effect of proline and 2,4-D on callus formation; (A) vigorous growth of callus in hypocotyls, (B) little or no significant callus on cotyledons, (C-E) decreased growth of calli on hypocotyles and (F-H) cotyledons with gradual increase of 2,4-D from 0.5 mg l^{-1} to 2.0 mg l^{-1}

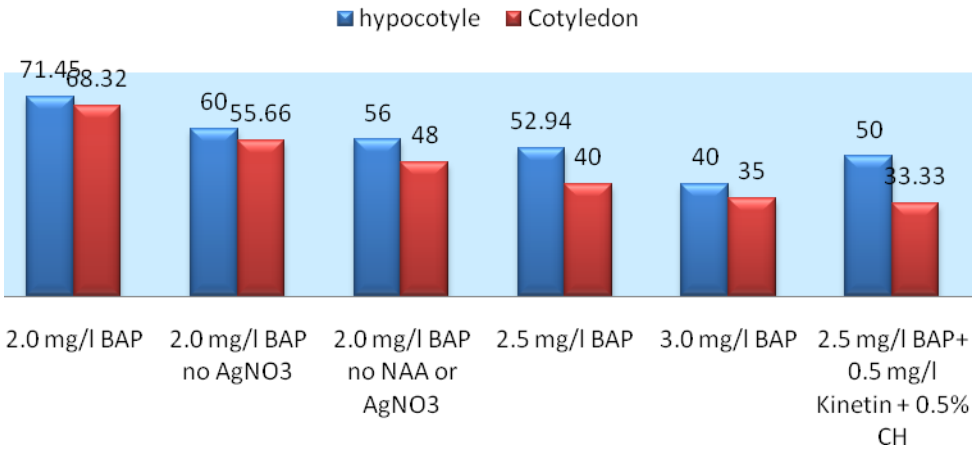

Fig.4. Percentage of shoot formation on BAP containing media from calli derived from hypocotyls and cotyledons of BARI-13 (in each case, 0.1 mgl^{-1} NAA and 5.0 mgl^{-1} $AgNO_3$ were used)

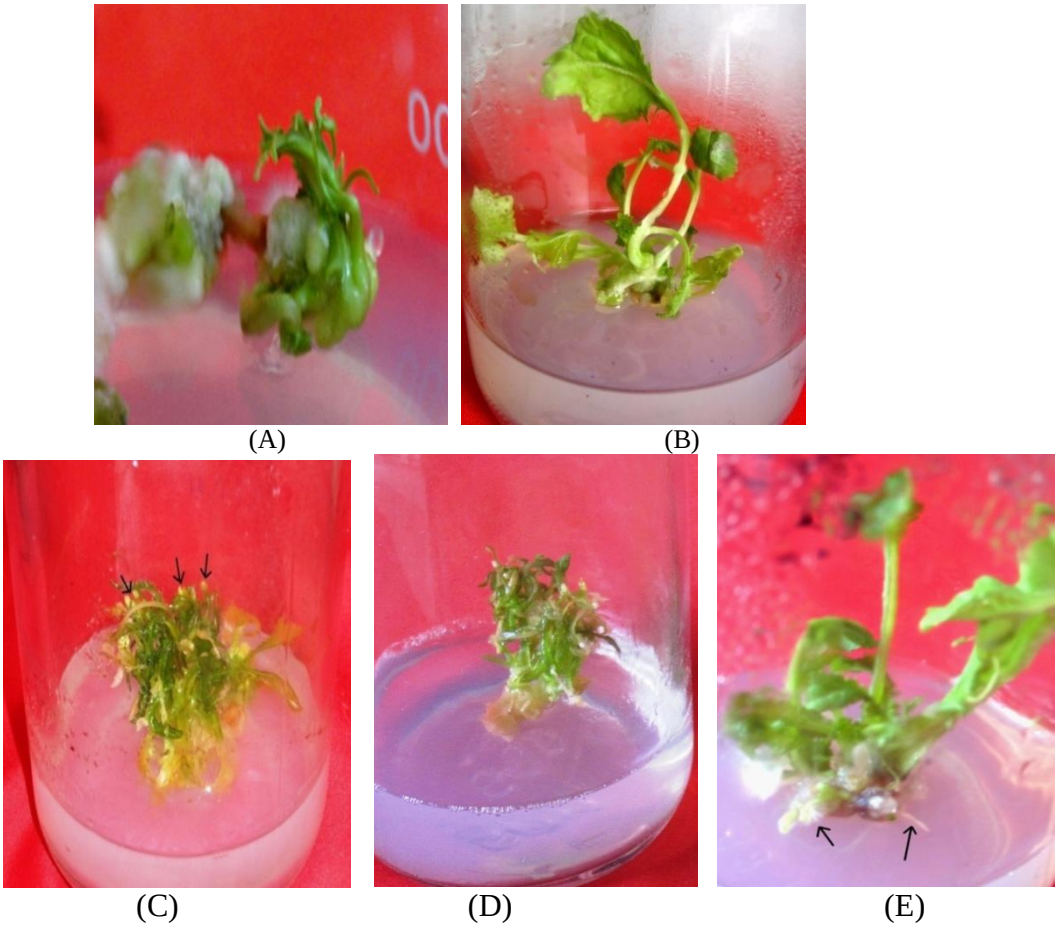

Fig. 5.: Different stages of *in vitro* regeneration of BARI-13: Initiation of shoots from callus from BARI-13 hypocotyl in medium supplemented with 2.0 mg l^{-1} BAP, 0.1 mg l^{-1} NAA, and 5.0 mg l^{-1} $AgNO_3$, (B) Development of shoots on the same medium, after 35 days (C) *In vitro* flowering (black arrows) from BARI-13 plantlets on the mentioned medium, (D) IBA having no effect on root development and (E) Initiation of root at the base of the *in vitro* regenerated shoots of BARI-13 on medium supplemented with 2.5 mg l^{-1} BAP and 0.5 mg l^{-1} NAA, after 10 days.

Table 1. Percentage of response of hypocotyl and cotyledon explants of BARI-13 on different callus induction media

Media	Percentage of responsive Hypocotyle explants	Callus type and color	Percentage of responsive Cotyledonary explants	Callus type and color
0.5 mgl^{-1} 2,4-D	100.00	Dark green, vigorous growth	91.00	Dark green
1.0 mg l^{-1} 2,4-D	90.91	Green compact	51.52	Green compact
2.0 mg l^{-1} 2,4-D	86.21	Green	64.62	Granular
0.5 mgl^{-1} BAP	100.00	Green and vigorous	91.00	Dark green
0.0 mgl^{-1} BAP	76.47	Tight , whitish	63.64	Loose white
0.5 mgl^{-1} IAA	80.00	White	80.00	White
0.5 mgl^{-1} IAA + 0.5 mgl^{-1} IBA	81.00	Loose white	81.00	Loose white
0.0 mgl^{-1} $AgNo_3$	86.49	Greenish, compact	73.91	Light green
0.5 mgl^{-1} Proline	92.16	Greenish, compact	62.50	Greenish
0.1% CH	100.00	Whitish green	87.50	Whitish green

3. RESULTS AND DISCUSSIONS

3.1 Hormonal and additives' effect on callogenesis

The callus induction media used in the experiment were supplemented with 3% (w/v) sucrose and were solidified with 0.8% (w/v) agar. The percentage of response of the hypocotyls and cotyledonary explants are given in Table 1. In all cases, better calli were formed from hypocotyls than cotyledons.

The weight of cotyledonary callus was the highest in the medium supplemented with 0.5 mg l^{-1} 2, 4-D , 0.5 mg l^{-1} BAP and 0.5 mg l^{-1} AgNO3 (Fig. 1). The percentage of callus formation in cotyledon was also the highest (91%) on that medium. So, for callus induction in cotyledonary leaves with petiole, this medium was found to be effective. BARI-13 gave slightly increased percentage of explants response when concentration of 2, 4-D was raised to 2. 0 mg l^{-1} (Table 1), but the size calli were very negligible. Medium without BAP and AgNO3 produced very poor callus for both hypocotyls and cotyledons (Fig.2 A, B), whereas the addition of BAP and AgNO3 increased the callus initiation and growth (Fig. 2 C, D).

In case of hypocotyls, 100% response was observed on medium supplemented with 0.5 mg l^{-1} 2, 4-D, 0.5 mg l^{-1} BAP and 0.1% CH (Table 1). Medium supplemented with 0.5 mg l^{-1} proline was also effective to induce a good percentage of responsive hypocotyl explants where callus was initiated and large and vigorously developed all over the hypocotyls (Fig. 3A). On the contrary, a very insignificant callus was formed in cotyledons on proline supplemented medium (Fig. 3 B). The highest average weight of hypocotyl callus achieved that grew on MS medium containing 0.5 mgl^{-1} 2,4-D, 0.5 mgl^{-1} BAP and 0.5 mgl^{-1} 2,4-D $AgNO_3$. Average weight of hypocotyl calli was similar when proline was used as additive. Increasing 2, 4-D concentration dramatically reduced callus growth on both hypocotyls and cotyledons (Fig. 3 C-H). Williums. *et al.* [14] reported the death of callus from hypocotyl explants shortly after withdrawal of $AgNO_3$. The use of AgNO3 was described to promote the growth of the callus by Williums *et al.* [14] that supports the findings of the current investigation.

3.2 Combinatorial effect of PGRs on shooting

For the induction and development of multiple shoots from calli obtained from hypocotyls and cotyledonary leaves with petioles of BARI-13 were inoculated in MS medium supplemented with BAP. The effects of NAA, $AgNO_3$, Kinetin and CH were also evaluated. BARI -13, the best percentage of shooting from hypocotyls callus (71.45%) was obtained in medium supplemented with 2.0 mg l^{-1} BAP, 0.1 mg l^{-1} NAA and 5.0 mg l^{-1} $AgNO_3$ (Fig. 5 A). On the same medium, cotyledonary calli also gave better shoot initiation (68.32%). *In vitro* flowering (Fig 5. C) was obtained in this medium but the flowers were smaller than *in vivo* flowers. No flower inducing hormone was used and this result supports that the endogenous factors of the shoots can create the environment for flowering that may include flowering hormones. About 14% of hypocotyl calli in this medium were found to produce roots after 12 days but they did not develop and were not in direct connection with the initiated shoots. The increase of BAP to 3.0 mg l^{-1} and NAA to 0.5 mg l^{-1} decreased the percentage of shoot initiation (Fig. 4). The callus in that case turned into green compact and finally died. Higher percentage of shoot formed from BARI-13 hypocotyl calli than that of cotyledons. Similar report was provided by Ali *et al.* [15] and Khan *et al.* [12] that supports the present investigation.

When kinetin was added to regeneration medium, hypocotyls calli became large compact but no significant shoot was initiated. The callus obtained from medium supplemented with proline produced better shoots. Medium supplemented with 0.5% CH was found to give the lowest percentage of shoot initiation and development from cotyledonary calli. CH has been used previously for both callus induction and regeneration [16]. Callus induction *in vitro* and plant regeneration is dependent on many factors including the genotype of the variety as well as composition of the culture medium, the type, combination and concentration of different PGRs. Shooting that was influenced by combinations of BAP and NAA, is supported by Hachey *et al.* [17]. *In vitro* flowering of the plant was recorded for BARI-13. The flowers were smaller than the *in vivo* flowers. The external factors like photoperiod and temperature had their effects on flowering that is supported by Stephen and Jayabalan [18]. The variation in response between hypocotyls and cotyledon explants found in current investigation is also reported by Minoru and Thomas [19].

3.3 Suitable hormonal supplements for rooting from the in vitro regenerated shoots

To get a complete plantlet, root formation is an obligatory step. Spontaneous root generation occurs sometimes on MS medium with hormonal supplements for the induction of shoots but plantlets with these roots were not found to be efficient to thrive in soil. Therefore, independent media supplemented with NAA and IBA were used to induce and develop roots. BARI-13 initiated and produced sufficient root system on medium containing 2.5 mg l^{-} NAA and 0.5 mg l^{-} BAP (Fig. 5 E). But when IBA was used at a concentration of 1.0 mg $l^{-1,}$ *in vitro* regenerated shoots produced no root (Fig. 5 D) and the plantlet became yellowish and finally died.

4. CONCLUSIONS

Increasing 2, 4- dichlorophenoxyacetic acid (2,4-D) from 0.5 mgl^{-1} to above and 6, benzyl amino purine (BAP) concentration from 2 mg^{-1} to 3 mg^{-1} and NAA from 0.1 mg^{-1} to 0.5 mg^{-1} decreased callus and shoot formation in BARI-13, respectively. Roots were obtained when naphtalene acetic acid (NAA) was used. Effects of kinetin, $AgNO_3$, casein hydrolysate (CH), proline, indole 3-butyric acid (IBA), etc were also seen in different stages of regeneration. The protocol can be used as an *in vitro* regeneration system which can be transmitted in the field in future.

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