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Genotype dependent influence of phytohormone combination and subculturing on micropropagation of sugarcane varieties

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Eight varieties of sugarcane, viz. Co.89003, Co.91010, Co.96258, Co.97017, Co.P84211, Co.P84212, Co.P90223, and Co.S767, were successfully established and micropropagated using shoots tips as explants. Four combinations of phytohormone, having NAA (1 mg/L) at fixed conc. and two cytokinins, i.e. BAP and Kn, at varying conc., were used; these were designated as A_1 (1 mg/L BAP + 2 mg/L Kn); A_2 (1 mg/L BAP + 3 mg/L Kn); A_3 (3 mg/L BAP + 2 mg/L Kn) and A_4 (6 mg/L BAP and 1 mg/L Kn). All the varieties were successively sub cultured up to 8th passage. Maximum percentage of explant establishment (80-100%) was observed for all the varieties, except Co.P.84212 and Co.P. 90223 (50-60%). No influence of genotype was found on the days required for side bud induction, average length of shoots from side bud, and number of days for multiple shoots when explants were placed in various phytohormone combinations. However, individual varieties showed preference for higher or lower conc. of cytokinins, i.e. growth of shoots in varieties Co.89003, Co.91010, Co.96258, Co.97017, Co.P84212 and Co.P.90223 was better in A_1 and A_2 hormone profile, while that of Co.91010 and Co.S767 in A_3 and A_4 hormone profile.

Keywords: genotype, micropropagation, phytohormone combinations, *Saccharum*

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Introduction

Sugarcane (*Saccharum officinarum*) is one of the major cash crops grown extensively all over the world. India is the second largest producer of sugarcane next to Brazil. Presently, about 4 m ha of land is under sugarcane with an average yield of 70 tonnes/ha. The sugarcane production of India was reported to be 201 lakh tonnes in the year 2002-2003, which declined to 170 lakh tonnes in the year 2003-2004. Thus, in the tropical regions of the country, sugarcane yield levels have been stagnating and even decreasing over the past few years. Hence, to reverse this trend and sustain a higher cane yield levels, there is an urgent need to increase cane productivity without further expansion in area. It is in this context that several programmes, such as the introduction of new high yielding varieties, changing the planting and harvesting season to optimize yields, utilization of improved ratoon management practice, adoption of crop management systems to minimize yield losses, fertilizer management for optimum results, use of quality seeds, etc., have been recommended and practiced. Among these, introduction of new high yielding varieties is the most cost-effective method for enhancing productivity.

Availability of quality seeds, free of pests and pathogens both for existing and newly released varieties is a major constraint. Introduction of a new variety takes several years due to low rate of multiplication (normally 1-10). However, micro-propagation technology bears very high potential for rapid clonal multiplication: it can be up to 1 million plants in a year from a single explant[1]. Further, sugarcane being a long duration crop is also prone to incipient diseases, which are difficult to monitor in field and, hence, get transferred to the next generation through seed. Furthermore, it is difficult to detect off type through early morphological validation of the crop. Traditional method of cane cultivation using three budded set requires large quantity of seed, which is costly, time consuming and land demanding. Such techniques of vegetative propagation based on cuttings are now outdated perceiving the advantages of micropropagation, which promises profuse clonal multiplication of plants with in a short time span and in a limited space with high output[1,2] in any season.

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Micropropagation recapitulates morphological and developmental events that occur in the field and takes place in the absence of vascular connections with the mother plant[3]. Following appropriate induction of side bud, the somatic cells can generate a complete plant, since they contain a complete set of genetic information[4]. The competence of cells, tissues and organs in response to specific environmental or chemical signals may be the starting point to new developmental programmes, leading to *in vitro* propagation[5]. Although micropropagation depends on genotype of the plant, but exogenous growth regulators are important for the expression of this capacity and is indispensable to shoot organogenesis[6].

Therefore, the present work deals with *in vitro* propagation and multiple shoot production of eight different genotypes of *S. officinarum*. Various parameters, like explant establishment, side bud induction and shoot regeneration, were studied for standardization of micropropagation strategy of high adapted varieties of Uttar Pradesh and Uttaranchal States of India.

Materials and Methods

Selection of Genotypes

A total of 8 varieties of sugarcane, viz. Co. 89003, Co. 91010, Co. 96258, Co. 97017, Co.P 84211, Co.P 84212, Co.P 90223 and Co.S 767, were chosen on the basis of their yield characters. Five sets of canes of each variety were brought from fields at a regular interval of one month from September to January and named as lot nos I, II, III, IV, and V. A recently formed tissue with quiescent bud was processed from adult plants, growing in Crop Research Centre, Pantnagar (Uttaranchal), India, and used as an explant.

Establishment of Explant

The explants were thoroughly washed in tap water using a liquid detergent Tween 20 (10%, v/v) ascorbic acid (0.2 %w/v), bavistine (0.1% v/v), citric acid (0.4% w/v), and streptomycin sulphate (0.1% w/v) for ½ h. Subsequently, excised explants were sterilized with alcohol (4 min) and given an intermittent wash of 5 min in distilled water before culture. The explants were cultured onto solid Murashige and Skoog's (MS) medium[7] supplemented with 0.8% agar, 3% sucrose and 0.5 ppm of naphthalene acetic acid (NAA), benzyl aminopurine (BAP) and kinetin (Kn) for establishment. The *p*H of the media was adjusted 5.7 prior to autoclaving at a pressure 1.06 kg/cm^2 for 25 min. To overcome the problem of phenolic oxidation, explants were transferred to fresh medium of the same composition supplemented with 0.3% soluble PVP every fortnightly[8]. All cultures were incubated at 25 ± 2^{o}C under l6 h photoperiod to 1000 lux provided by white fluorescent lamps. All experiments were repeated thrice with at least 20 replicates for each variety.

Table 1—Selection of hormonal regimes based on their total cytokinin

Media/ Nomenclature	NAA mg/L	BAP mg/L	Kn mg/L	Reference
(A_1)	1	1	2	Shukla *et al* (1996)
(A_2)	1	1	3	Lal *et al* (1994)
(A_3)	1	3	2	Lal *et al* (1994)
(A_4)	1	6	1	Sreenivasan *et al*, (1992); Lal *et al*, (1999)

Shoot Induction and Multiplication Medium

For shoot induction, excised-sprouted explants were placed onto MS medium supplemented with 1 mg/L NAA + 1 mg/L BAP + 2 mg/L Kn for a period of 30 d. Multiple shoots were then transferred to four phytohormone combinations designated as A1, A2, A3 and A4, used by various workers (Table 1). These phytohormone combinations had increasing concentration of cytokinins, BAP and Kn.

Root Induction Medium

For root induction, shoots (76 cm) were excised and transferred into liquid MS medium supplemented with 5 mg/L of sucrose. Plantlets with well developed roots and expanded leaves, were removed from the culture medium and, after washing of the roots with autoclaved tap water, were transferred to plastic bags containing sand:soil:FYM (1:1:1) mixture. These plantlets were subjected to acclimatization by covering them with polyethylene bags and were kept in green house at 90% relative humidity. Successfully hardened plantlets were eventually established in natural soil.

Results and Discssion

Effect of Establishment Percentage

Varieties, Co.P 84212 and Co.P 90223 showed significantly lower establishment percentage of cane (60-50%) as compared to other varieties (80-100%; Table 2). Incidentally, lower establishment varieties are much more prone to contamination than the others in all seasons, indicating that these varieties are

having in-built dormant living contaminating entities which are unable to over come by surface sterilization. The reason for this could be that these varieties have highest sugar and least fiber content. However, except for few instance, no effect of season was observed on the percentage establishment. The seasonal variation on percentage establishment could be presumed due to climatic conditions, especially high humidity in varying season.

Effect on Side Bud Sprouting

Micropropagation in sugarcane is perhaps something similar to tillering, where a shoot base is a stunted shoot and auxiliary buds get sprouted in largest frequencies in a cascade form, i.e. quiescent bud of newly formed shoot also sprouts to give additional shoot. Alternately, the base of shoot is like a differentiating mass that gives rise to shoot buds through differentiation. It was expected that varieties differ in their ability in the rate of sprouting and growth of the quiescent axillary buds, and influence the rate of micropropagation. Hence, the days required for visible sprouting of the bud in the explants and its length after a month (30 d) were recorded for each of the genotype studied in the present investigations.

All genotypes selected took nearly the same number of days for side to sprout, i.e. 8-10 d. Genotypically, not much variation was observed for this trait. The change in season also did not influence the rate of sprouting (Table 3).

Table 2 — Effect of genotype on explant establishment in different months

Variety\Set	Percentage establishment*				
	September	October	November	December	January
	I	II	III	IV	V
Co. 89003	95.00	93.30	90.00	91.00	80.00
Co. 91010	88.00	92.50	89.60	86.50	86.00
Co 96258	100.00	93.30	96.00	98.00	97.80
Co. 97017	95.20	86.60	82.50	90.00	76.90
Co. P 84211	100.00	100.00	92.00	80.00	69.60
Co. P 84212	51.55	91.41	55.00	67.10	78.00
Co. P 90223	62.50	95.20	55.60	58.00	68.00
Co.S 767	93.30	76.00	82.50	55.00	79.40

*Mean of twenty different independent replicates

Table 3 — Effect of genotype on side bud sprouting in different months

Variety\Set	Days required for side bud induction (Mean ± SE)				
	September	October	November	December	January
	I	II	III	IV	V
Co. 89003	10.0 ± 0.25	11.0 ± 0.25	10.0 ± 0.25	10.0 ± 0.0	10.0 ± 0.0
Co. 91010	9.0 ± 0.25	8.0 ± 0.25	10.0 ± 0.25	10.0 ± 0.50	11.0 ± 0.47
Co. 96258	8.0 ± 0.47	10.0 ± 0.25	11.0 ± 0.00	11.0 ± 0.28	12.0 ± 0.00
Co. P 84211	8.0 ± 0.25	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0
Co. P 84212	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0
Co. P 90223	11.0 ± 0.25	12.0 ± 0.028	10.0 ± 0.25	11.0 ± 0.28	11.0 ± 0.28
Co.S 767	10.0 ± 0.00	10.0 ± 0.00	11.0 ± 0.47	11.0 ± 0.47	10.0 ± 0.47

SE:Standard error

Days for Multiple Shoot Induction and Average Length of Shoots

In order to investigate the influence of the genotypes, the time required for multiple shoot induction and variation in average number of shoots per transfer were recorded for all the varieties after they were transferred from establishment media to a common proliferating media. After a bunch of shoots have proliferated for one month in the common proliferating media, the shoots were transferred to various phytohormone combinations as mentioned in Table 1. Bunch of shoots were allowed to proliferate for one month to bring all the cultures at one stage. Due to variation in genetic makeup of the plants, it was not expected that a single combination of phytohormones will suite to all the varieties. Thus, average length of cultures during subsequent transfers in all the four combinations of phytohormones was recorded. Twenty replicates of each variety were taken and their readings were recorded and statistically analyzed.

The average lengths of leaves/shoots at 30th day have been summarized in Table 4; it ranged from 5.3 cm (Co.P 90223) to 11.2 cm (Co.P 84211). Variations so observed seem to be influenced by the genotypic difference, as the differences were seen under a defined temperature and light, and were not influenced by the months, which are a representative of differences in geogravity of earth in different seasons. However, no effect of genotypes was evident on the days required for multiple shoot induction in various hormone profiles; an average of 24-27 d was required for multiple shoot induction (Table 5).

Table 4 — Effect of genotype dependent seasonal variation on average length of leaves/shoots emerging from side bud after thirty days of inoculation

Variety\Set	Average length of leaves/shoots emerging from side bud after 30 days (cm) (Mean ± SE)				
	September	October	November	December	January
	I	II	III	IV	V
Co. 89003	7.0 ± 0.75	7.0 ± 0.47	7.5 ± 0.13	7.4 ± 0.22	8.0 ± 0.40
Co. 91010	7.2 ± 0.85	7.2 ± 0.40	8.1 ± 0.13	7.4 ± 0.11	7.5 ± 0.19
Co. 96258	6.2 ± 0.85	7.6 ± 0.91	6.3 ± 0.64	7.5 ± 0.13	7.3 ± 0.10
Co. 97017	8.0 ± 0.64	8.0 ± 0.70	8.4 ± 0.70	8.2 ± 0.62	8.5 ± 0.85
Co. P 84211	10.4 ± 0.15	11.2 ± 0.10	8.4 ± 0.10	9.0 ± 0.50	9.5 ± 0.17
Co. P 84212	8.1 ± 0.85	8.2 ± 0.14	8.3 ± 0.70	7.8 ± 0.85	7.2 ± 0.10
Co. P 90223	5.8 ± 0.57	5.3 ± 0.10	6.5 ± 0.13	4.3 ± 0.85	4.6 ± 0.10
Co.S 767	6.2 ± 0.85	7.5 ± 0.16	7.4 ± 0.16	6.6 ± 0.20	6.5 ± 0.17

SE: Standard error

Table 5 — Days required for multiple shoot induction in various hormone profiles (Mean ± SE)

Variety	A1	A2	A3	A4
Co. 89003	27.0 ± 0.35	27.0 ± 0.42	27.0 ± 0.05	24.0 ± 1.6
Co. 91010	25.2 ± 0.62	25.0 ± 0.48	26.0 ± 0.21	21.0 ± 1.2
Co. 96258	22.0 ± 0.61	19.0 ± 0.28	27.0 ± 0.21	20.0 ± 1.78
Co. 97017	25.0 ± 0.64	24.0 ± 0.47	26.0 ± 0.39	21.0 ± 0.28
Co. Pant 84211	27.0 ± 0.27	27.0 ± 0.43	27.0 ± 0.32	22.0 ± 0 3.4
Co. Pant 84212	24.0 ± 0.21	27.0 ± 0.47	20.0 ± 0.28	25.0 ± 1.2
Co. Pant 90223	26.8 ± 0.42	27.0 ± 0.60	26.0 ± 0.32	26.0 ± 2.2
Co.S 767	26.0 ± 0.20	26.0 ± 0.21	23.0 ± 0.45	24.0 ± 1.6

SE: Standard error

Average Number of Shoots Induced and Length of Shoots in Various Hormone Profiles

Not much variation was observed in the average number of shoots induced in various hormone profiles (Table 6). However, it is evident from Table 7 that, as the subcultures are transferred from a common proliferating media to the test phytohormone combinations A1 and A2, a regular increase in the length of shoots was observed with the increase in the number of subcultures for all the varieties, except Co.91010 and Co.S767 where converse was found true. However, an absolutely reverse trend was observed with A3 and A4 hormone profiles (Table 8). It was observed that those varieties, which showed an increasing trend in A1 and A2 hormone profile, showed a decreasing trend in A3 and A4. Similarly, varieties Co.91010 and Co.S767, which had shown decreasing trend with A_1 and A_2 hormone profiles, showed an increasing trend in A3 and A4 hormone profiles.

Thus, the genotypes showed a varied sensitivity to various phytohormone combinations. It is also from the above studies that the growth parameters like establishment percentage and the length of the sprouted shoots are independent of any seasonal constraints and depends entirely on the type of genotype taken into account[9,10]. The effect of genotype is also clearly evident when we study the effect of average shoots length during progressive subcultures in A1, A2, A3 and A 4 hormone profiles (Tables 7 & 8), where some varieties preferred a low cytokinin concentration for robust growth, while other varieties required high cytokinin concentration. It is an established fact that phytohormones basically influence the process of cell division, cell-elongation

Table 6 — Number of shoot induced in various hormone profiles (Mean ± SE)

Variety	A1	A2	A3	A4
Co. 89003	124.5 ± 0.84	99.4 ± 1.5	109.0 ± 1.10	94.0 ± 5.2
Co. 91010	115.0 ± 0.45	96.2 ± 0.34	111.0 ± 1.2	87.0 ± 6.3
Co. 96258	91.3 ± 0.39	111.0 ± 0.34	95.8 ± 0.29	99.0 ± 0.39
Co. 97017	114.6 ± 10.0	118.6 ± 1.1	110.0 ± 1.05	72.0 ± 6.2
Co. Pant 84211	188.5 ± 0.32	113.9 ± 1.2	104.7 ± 0.63	98.0 ± 6.7
Co. Pant 84212	76.4 ± 0.30	85.1 ± 0.86	81.2 ± 0.51	76.0 ± 6.2
Co. Pant 90223	115.5 ± 1.1	168.4 ± 5.0	107.6 ± 1.7	100.2 ± 7.8
Co.S 767	130.0 ± 0.86	169.7 ± 0.31	126.0 ± 0.5	57.0 ± 0.37

SE: Standard error

Table 7—Average shoots length during progressive subcultures in medium A1 and A2 hormone profiles (Mean ± SE)

Variety	Average length of shoots in progressive subcultures (Mean ± SE)				Average length of shoots in progressive subculture (Mean ± SE)			
	T_2	T_4	T_6	T_8	T_2	T_4	T_6	T_8
Co. 89003	5.3 ± 0.32	6.2 ± 0.44	6.4 ± 0.42	7.9 ± 0.47	6.2 ± 0.80	6.0 ± 0.06	7.2 ± 0.06	8.6 ± 0.09
Co. 91010	**7.2 ± 0.66**	6.8 ± 0.37	6.3 ± 0.46	**5.4 ± 0.32**	**8.2 ± 0.07**	8.4 ± 0.04	6.9 ± 0.03	**4.3 ± 0.05**
Co. 96258	5.8 ± 0.38	6.3 ± 0.79	7.2 ± 0.57	8.0 ± 0.76	5.1 ± 0.28	6.6 ± 0.71	7.8 ± 0.48	8.5 ± 0.76
Co. 97017	5.1 ± 0.33	5.4 ± 0.63	6.8 ± 0.57	6.9 ± 0.78	6.6 ± 0.07	6.9 ± 0.10	7.6 ± 0.15	8.6 ± 0.08
Co. Pant 84211	5.0 ± 0.22	5.4 ± 0.42	6.9 ± 0.38	7.1 ± 0.36	4.7 ± 0.13	5.7 ± 0.05	6.5 ± 0.06	7.4 ± 0.06
Co. Pant 84212	4.2 ± 0.43	4.7 ± 0.45	5.8 ± 0.32	7.2 ± 0.52	4.4 ± 0.07	5.6 ± 0.08	6.2 ± 0.11	7.4 ± 0.07
Co. Pant 90223	5.7 ± 0.51	5.0 ± 0.52	5.7 ± 0.32	6.2 ± 0.77	6.3 ± 0.12	6.5 ± 0.06	6.6 ± 0.10	8.4 ± 0.15
Co.S 767	**6.2 ± 0.36**	7.9 ± 0.66	5.7 ± 0.68	**4.9 ± 0.33**	**9.0 ± 0.04**	8.2 ± 0.03	7.0 ± 0.02	**4.7 ± 0.02**

SE: Standard error

and cell-differentiation, and they regulate and integrate the overall growth development and reproduction in plants. Auxins and cytokinins are known to act synergistically and/or antagonistically to regulate these processes of plant growth[11,12], which could be attributed to the differences in auxin/cytokinin signaling pathways associated with plant growth in different genotypes as indicated by the findings of the present study. The representative steps of micropropagation have been demonstrated in Figs 1 (establishment of explants), 2 (multiplication) and 3 (hardening).

Micropropagation protocol standardized here did not involve callus, which decreases the probability of somaclonal variations. Thus, the genotype dependent choice of a suitable hormone combination is well justified in micropropagation of sugarcane genotypes and in genetic manipulations. We can also conclude that micropropagation of sugarcane plants is dependent on the genotypes of mother plant and the phytohormone combinations used during culture, and

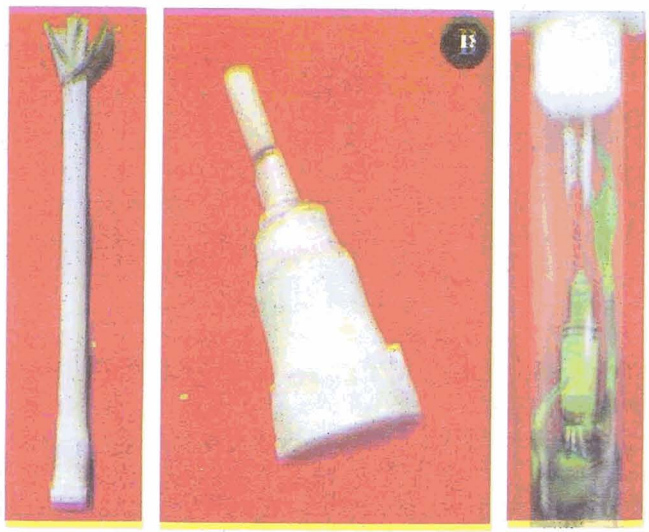

Fig. 1—(A-C) Establishment of explant: (A) The explants were taken from the actively growing shoot tops of 5-8 months old sugarcane plants; (B) The excised pieces, containing a bud, were used as explants in the present investigation; (C) The excised pieces, containing a bud, placed in establishment media in the test tube showing side bud induction.

Fig. 2—(A-G) Multiplication: (A) The sprouted bud was excised aspectically and placed in multiplication media. First, a piece of explant along with newly emerged shoot was excised. (B) Subsequently, the shoot was excised from the explant and multiple injuries were given at the base and were transferred to the same proliferating medium. (C) A new developing bud been seen from the injured place (2nd transfer). (D) The developed bud elongates to form a new shoot. As new leaves emerge from these buds, the old shoot is decapitated. This operation induces emergence of new shoots from the base. (E-G) These were allowed to incubate till a sufficient number of shoots appeared from the base.

Fig. 3—(A-G) Hardening: (A) Plants were taken out from culture bottles; (B-E): Hardening of *in vitro* raised plants in plotting mixture of soil + sand + FYM (1:1:1); (F-G) All plants survived and tillered profusely in the field.

Table 8 —Average shoots length during progressive subcultures in medium A3 and A 4 hormone profiles (Mean ± SE)

Variety	Average length of shoots in progressive subcultures (Mean ± SE)				Average length of shoots in progressive subculture (Mean ± SE)			
	T_2	T_4	T_6	T_8	T_2	T_4	T_6	T_8
Co. 89003	8.3 ± 0.06	6.3 ± 0.06	5.6 ± 0.06	4.3 ± 0.06	7.2 ± 0.03	7.3 ± 0.04	4.5 ± 0.05	3.4 ± 0.06
Co. 91010	**4.4 ± 0.06**	4.5 ± 0.05	5.3 ± 0.08	**6.7 ± 0.18**	**2.9 ± 0.15**	3.3 ± 0.08	4.2 ± 0.05	**7.3 ± 0.6**
Co. 96258	8.3 ± 0.06	7.3 ± 0.05	4.4 ± 0.07	4.0 ± 0.11	7.2 ± 0.06	6.2 ± 0.10	4.9 ± 0.20	3.5 ± 0.10
Co. 97017	8.5 ± 0.05	7.5 ± 0.05	5.2 ± 0.06	4.9 ± 0.04	8.2 ± 0.04	6.3 ± 0.06	5.5 ± 0.04	4.3 ± 0.06
Co. Pant 84211	8.5 ± 0.07	6.3 ± 0.18	5.4 ± 0.08	4.5 ± 0.06	7.2 ± 0.09	7.2 ± 0.05	4.2 ± 0.05	3.6 ± 0.06
Co. Pant 84212	6.2 ± 0.04	5.2 ± 0.04	4.8 ± 0.02	4.1 ± 0.04	5.2 ± 0.06	4.3 ± 0.04	3.3 ± 0.05	2.3 ± 0.07
Co. Pant 90223	7.8 ± 0.10	7.4 ± 0.09	5.2 ± 0.03	4.6 ± 0.05	8.3 ± 0.07	7.3 ± 0.08	5.1 ± 0.10	4.4 ± 0.07
Co.S 767	**4.0 ± 0.08**	5.4 ± 0.07	6.3 ± 0.05	**7.5 ± 0.05**	**4.6 ± 0.04**	5.0 ± 0.07	5.5 ± 0.09	**7.8 ± 0.18**

SE: Standard error

to obtain rapid clonal propagation, it is essential that instead of using a single phytohormone combination for propagation of all the genotypes under culture, phytohormone combinations should be optimized for each and every genotype individually.

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