SOMATIC EMBRYOGENESIS IN *Citrus sinensis*, *C. reticulata* AND *C. nobilis* x *C. deliciosa*

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ABSTRACT: Most of the plant regeneration processes in citrus, through tissue culture, involve indirect somatic embryogenesis. The optimization of these processes is important for the development of *in vitro* plant improvement and micropropagation studies. Studies to evaluate the effect of different carbohydrates in somatic embryogenesis were conducted using calli from 'Ponkan' mandarin (*Citrus reticulata*, Blanco), 'Cravo' mandarin (*C. reticulata*), 'Itaboraí' sweet orange (*C. sinensis* L. Osbeck.), 'Valencia' sweet orange (*C. sinensis*) and 'Kinnow' mandarin (*C. nobilis* Loureiro x *C. deliciosa* Tenore). The culture medium used was MT supplemented with sucrose, galactose, glucose, maltose or lactose with the following concentrations of 18, 37, 75, 110, and 150 mM. The culture medium used for the maturation of somatic embryos had 0, 15, 29, 44, 58 and 73 mM of sucrose, in presence or absence of 0.5 g L^{-1} of activated charcoal. Seventy-three mM of sucrose with 0.1 mg L^{-1} of GA_3 in the presence or absence 0.5 g L^{-1} of activated charcoal was also tested. Overall, the carbohydrates galactose or lactose induced a higher number of somatic embryos. Sucrose concentrations of 58 and 73 mM generated a higher number of plantlets from mature embryos of 'Ponkan' mandarin and 'Valencia' sweet orange.
Key words: callus, carbohydrate, citrus, somatic embryo

EMBRIOGÊNESE SOMÁTICA EM *Citrus sinensis*, *C. reticulata* E *C. nobilis* x C. deliciosa

RESUMO: A maioria dos processos de regeneração de plantas em citros por cultura de tecidos envolve embriogênese somática indireta. A otimização desse processo é importante para o desenvolvimento de trabalhos de melhoramento *in vitro* e micropropagação. Realizaram-se estudos em calos de tangerina 'Ponkan' (*Citrus reticulata* Blanco), tangerina 'Cravo' (*C. reticulata*), laranja 'Itaboraí' (*C. sinensis* L. Osbeck.), laranja 'Valência' (*C. sinensis*) e tangerina 'Kinnow' (*C. nobilis* Loureiro x *C. deliciosa*, Tenore) visando avaliar o efeito de diferentes carboidratos na embriogênese somática. Utilizou-se o meio MT acrescido de sacarose, galactose, glicose, maltose e lactose nas concentrações de 18, 37, 75, 110 e 150 mM. O meio de cultura utilizado para a maturação dos embriões somáticos continha sacarose nas concentrações de 0, 15, 29, 44, 58 e 73 mM, na presença ou não de 0,5 g L^{-1} de carvão ativado; 73 mM de sacarose e GA_3 na concentração de 0,1 mg L^{-1}, na presença ou não de 0,5 g L^{-1} de carvão ativado. O estímulo à embriogênese somática foi mais eficiente em meio de cultura suplementado com lactose e galactose. Quanto à maturação de embriões, os meios de cultura contendo 58 e 73 mM de sacarose foram aqueles que geraram um maior número de plantas em tangerina 'Ponkan' e laranja 'Valência'.
Palavras-chave: calo, carboidrato, citros, embrião somático

INTRODUCTION

The citrus production in the State of São Paulo, which corresponds to 80% of the Brazilian yield, was estimated in the harvesting season of 2000/2001 to be 347.7 million boxes (40.8 kg/box) (IEA, 2001). According to FAO (2001), this amount represents 1/3 of the world's production thus ranking Brazil as the top citrus producer in the world. Although Brazil being considered the production leader, the citrus industry of the state of São Paulo presented several problems in the last five years. The orchard yields are among the lowest in the world, approximately 20 ton ha^{-1}. The factors responsible for the low productivity are mainly, the low genetic potential of the cultivated varieties, inappropriate crop management and the threat of several pests and diseases, such as Citrus Canker, Citrus Variegated Chlorosis, Black Spot and Citrus Blight (Pompeu Jr., 1991; Fundecitrus, 2001).

Traditional genetic plant improvement offers limitations for the production of new varieties of scion and rootstocks, and the new varieties produced so far were

originated from natural selection and mutation. The first barriers met by researchers were related to the complex citrus biology, which has nucellar polyembryony (apomixis), high heterozygosis, autoincompatibility, interincompatibility and a long juvenile period (Grosser & Gmiter Jr., 1990). Therefore, for Brazil to keep the international citrus leadership, plants free of pathogens with a high genetic potential new varieties need to be produced, thus allowing for an increase in the yields. The tissue and cell culture started as an auxiliary technique, which were used in the plant genetic improvement of citrus species (Mourão Filho et al., 1992). Studies related to citrus in this field were initiated with the purpose of acquiring *in vitro* nucellar embryos from polyembrionic species to obtaining virus-free plants. Later, the production of nucellar calli intensified due to the possibility of obtaining a large number of somatic embryos (Cristofani, 1991).

Another strategy used for producing improved varieties of citrus, for scion as well as for rootstocks, is somatic hybridization. This technique consists of combining complementary parents with the purpose of transferring desired traits to new plants such as resistance to *Phytophthora*, Citrus Canker, Citrus Variegated Chlorosis, Blight and drought (Grosser & Gmitter Jr., 1990; Mourão Filho et al., 1996). However, the literature does not cite efficient protocols for the induction and culture of nucellar embryogenic calli for all of the citrus varieties. These calli can be obtained from aborted ovule culture, non-fertilized ovule, and whole fertilized ovules or from nucellus. The environmental conditions where the plant develops, its genotype, the age of the explants, the components of the culture medium and the growth conditions are factors that highly influence the induction of nucellar embryogenic calli (Grosser & Gmitter Jr., 1990).

Among the culture medium components, carbohydrates have a strong importance in inducing somatic embryogenesis and embryo culture. Sucrose is the most common carbohydrate utilized in tissue or cell culture and the majority of culture media have it as a sole energy source. The concentration of the carbohydrate is also important. Other carbohydrates have also induced the somatic embryogenesis. Studies conducted by Perez et al. (1998) revealed that lactose and maltose enhanced the frequency of somatic embryogenesis. Kochba et al. (1982) reported that galactose and lactose caused a significant induction of somatic embryogenesis in 'Shamouti' sweet orange (*C. sinensis* L Osbeck), and 'Villafranca' lemon (*C. limon* Burmann). Moreover, these carbohydrates, at 64 mM concentrations promoted a substantial increase in the number of embryos derived from calli of 'King' mandarin (*C. nobilis* Loureiro), and 'Navel' sweet orange (*C. sinensis*). According to Tomaz et al. (2001), galactose, lactose or maltose had a great effect on somatic embryogenesis for the lines of calli from 'Valencia' and Caipira sweet oranges (*C. sinensis*) and for the Cleopatra (*C. reshni* Hort. ex Tanaka) mandarin.

After the formation of the somatic embryos the following stage is the maturation of the embryos. At this stage, the culture medium should provide the embryo with a simulated water stress so the embryos can accumulate reserves, such as proteins and aminoacids, thus inciting its germination.

The first reports of *Citrus* somatic embryogenesis did not describe a treatment that favored the maturation of the embryos. These reports described that the embryos were transferred from the somatic embryogenesis induction medium straight to the germination medium, which normally contained gibberellic acid (GA_3). Kochba et al. (1974) documented that, under these conditions, most of the time a mass of callus was covering the embryo, thereby causing embryo oxidation and consequently the failure of embryo germination. Perez et al. (1998) observed that 1 mg L^{-1} GA_3 was not necessary in the culture medium for the maturation of citrus somatic embryos.

Kunitake et al. (1991) conducted studies with heart-shaped embryos, which were derived from protoplasts of 'Satsuma' (*Citrus unshiu* Marcovitch) mandarin. They were transferred to culture medium containing half strength of the salt concentration found in the original MT medium and supplemented with GA_3 (1 mg L^{-1}), sucrose (1%) and agar (0.2%). Their study revealed that after incubation for two months, only 5% of the embryos had a normal development with further plant regeneration and 50 to 60% showed an abnormal growth with a secondary embryogenesis.

Studies done by Tomaz et al. (2001) on the effect of activated charcoal on the germination of somatic embryos derived from the variety 'Seleta Vermelha' (*C. sinensis*) revealed that the number of non-germinated somatic embryos was much higher than the regenerated plantlets. This suggests that activated charcoal adsorbed compounds involved in the germination process.

Therefore, due to the importance of somatic embryogenesis for plant improvement *in vitro* (somatic hybridization), this study evaluated in five citrus varieties the effect of: a) different sources and concentrations of carbohydrates on somatic embryogenesis, and b) gibberellic acid (GA_3), sucrose and activated charcoal on the maturation of somatic embryos and further plant.

MATERIAL AND METHODS

Plant Material

The following varieties of citrus were used for the production of embryogenic calli: 'Ponkan' mandarin (*C. reticulata*), 'Cravo' mandarin (*C. reticulata*), 'Itaboraí' sweet orange (*C. sinensis*), 'Valencia' sweet orange (*C. sinensis*) and 'Kinnow' mandarin (*C. nobilis* x *C. deliciosa*, Tenore). The culture medium used was MT (Murashige

& Tucker, 1969) supplemented with 500 mg L^{-1} of malt extract and 50 g L^{-1} of sucrose. The growth conditions were under darkness and at 27 ± 2°C. The calli were subcultured to fresh medium every four weeks. The lines callus were derived from nucellar tissue and/or from non-fertilized ovules and were subcultured to fresh medium for approximately 12 months.

Source of carbohydrates on induction of citrus somatic embryogenesis calli

Each Petri dish received 50 mg of embryogenic callus and 1.0 mL of the same medium was dropped on top of each mass of cells. Through circular movement, the mass of cells was dissociated, thereby obtaining a homogeneous distribution of cells on the surface of the medium. The growth conditions were under indirect light (300 lux) with photoperiod of 16 h at 27 ± 2°C.

The experimental design was completely randomized with three replications and each one had one Petri dish with 50 mg of callus. The number of embryos developed per treatment was evaluated with the help of stereomicroscopy (Nikon 102).

Maturation and germination of somatic embryos

Somatic embryos at the cotyledonar stage were used to evaluate the effect of the treatment tested for embryo maturation and plantlet production. The embryos derived from calli of 'Valencia' sweet orange, as well as 'Cravo', 'Kinnow', and 'Ponkan' mandarins were obtained from the MT culture medium supplemented with 37 mM of lactose and 500 mg L^{-1} of malt extract. The embryos derived from calli of 'Itaboraí' sweet orange were obtained from the MT culture medium supplemented with 75 mM of lactose and 500 mg L^{-1} of malt extract.

Somatic embryos were transferred to solid culture media containing different concentrations of sucrose (0,15, 29, 44, 58 and 73 mM) with or without 500 mg L^{-1} of activated charcoal. They were also transferred to solid culture medium containing 73 mM of sucrose and 0.1 mg L^{-1} GA_3, in the presence or absence of 500 mg L^{-1} of activated charcoal. The experimental design was completely randomized with three replications per treatment. Each replication had one disposable Petri dish (100 ´ 15 mm) with five embryos. The growth conditions were at 27 ± 2°C and photoperiod of 16 h of light 300 Lux. After 15 days, the somatic embryos were transferred to solid culture medium containing 73 mM of sucrose and 0.1 mg L^{-1} of GA_3 and they were incubated at 27 ± 2°C with a photoperiod of 16 h of light 300 Lux. The number of somatic embryos germinated per treatment was evaluated five weeks later.

The results were evaluated by the analysis of variance and Tukey test to compare the mean (level of significance at 1%). The data obtained were transformed in $\sqrt{x+0,5}$.

RESULTS AND DISCUSSION

Source of carbohydrates on induction of citrus somatic embryogenesis calli

The development of somatic embryogenesis of citrus calli was observed, through a stereomicroscope, according to the different embryonic stages: globular, cotyledon, torpedo and heart-shaped. A high number of abnormal embryos were formed in all treatments, such as embryos with more than two cotyledons or with clustered cotyledons; with only one normal cotyledon; with fused cotyledons; embryos with branched apexes; oxidized embryos and embryos covered with callus.

Similar abnormalities were documented in the literature (Coelho, 1997). Perez et al. (1998) reported the presence of embryos at the globular stage and callus proliferation at the same time when the culture medium was supplemented with lactose or maltose, which indicated the absence of cell synchronism during the development of citrus somatic embryos.

Among the carbohydrates, galactose and lactose induced the highest number of somatic embryos for 'Ponkan' at 75 mM of lactose (Table 1). Similar responses were observed for the varieties 'Valencia' sweet orange, and 'Kinnow' mandarin, with the exception of the 'Cravo' mandarin, which had the lowest number of embryos. The best concentrations of lactose for 'Valencia', 'Kinnow' and 'Cravo' were 37 mM, 75 mM and 37 mM, respectively (Table 1). These results are similar to that documented by Kochba et al. (1982), who studied somatic embryogenesis of callus obtained from several varieties of *Citrus* and found that galactose or lactose was very effective at inducing the embryo formation. Also, according to them, galactose induces the production of ethylene and that high level of ethylene inhibits the biosynthesis of auxins, which favors the formation of somatic embryos in embryogenic calli. This fact might explain the responses observed in the citrus varieties mentioned above.

Ling & Iwamasa (1997) conducted studies on plant regeneration from embryogenic calli derived from six varieties of *Citrus*, which were cultured in MT medium supplemented with 5% of lactose, instead of sucrose. The data obtained from their studies showed that embryos were formed in all varieties even though the frequency, amount and quality of them varied depending upon the variety in study.

The favorable effect of galactose on somatic embryogenesis was verified by Tomaz et al. (2001), who reported that 110 mM of galactose caused a higher number of somatic embryos of callus derived from the Caipira sweet orange. On the other hand, Kunitake et al. (1991) observed that the formation of embryos from callus of 'Satsuma' mandarin was induced only when lactose, at the concentration of 15 mM, was the carbon source, while no embryos were formed when 15 mM of glucose or sucrose was used.

Benedito et al. (2000) found different responses on the induction of embryo formation when comparing different varieties of *Citrus*. They observed that the sweet orange varieties 'Bahia Cabula' and 'Rubi' produced 1.700 embryos per 50 mg of callus, while from the calli of 'Valencia,' and 'Orvalho de Mel' (*C. sinensis*) derived approximately 650 embryos. Their studies also showed that galactose was the carbon source that yielded the highest number of embryos, even though the concentration of galactose was different for each variety studied.

Sucrose was not very efficient at inducing somatic embryogenesis for the evaluated varieties (Table 1). Similar results were also documented by Kochba et al. (1982), who showed that concentrations higher than 32 mM of sucrose inhibited the formation of embryos in callus from the tested varieties of *Citrus*. Tomaz et al. (2001) found that in the presence of sucrose only a few somatic embryos were derived from the calli of the Caipira sweet orange and Cleópatra mandarin. Moreover, the inefficiency of sucrose was also observed by Mendes-da-Glória et al. (2000). They found that the induction of somatic embryos in calli derived from protoplast fusion of citrus was better when the culture medium used, EME (Grosser & Gmitter Jr., 1990), had sucrose replaced with 73 mM of maltose.

Maltose at concentration up to 75 mM induced the somatic embryogenesis on the callus from 'Itaboraí' sweet orange, while the induction effect of galactose was better at the concentration of 110 mM (Table 1). Finally, overall, lactose was not efficient in promoting somatic embryogenesis. This observation does not agree with the studies conducted by Singh et al. (1992), who reported that lactose at concentrations between 29 to 146 mM induced the somatic embryogensis in the callus of 'Shamouti' sweet orange.

Table 1 - The effect of different sources and concentrations of carbohydrates on somatic embryogenesis of citrus,Piracicaba, SP.

Concentration	18 mM	37 mM	75 mM	110 mM	150 mM
	Mean number of embryos per Petri dish				
Carbohydrate	'Ponkan' mandarin				
Sucrose	0 dA	0 dA	0 dA	0 cA	0 cA
Galactose	731.6 aA	440.1 bB	623.4 AB	701.6 aA	653.2 aA
Glucose	41.8 cA	42.3 cA	62.4 cA	43.8 bA	47.5 bA
Lactose	314.4 bC	820.9 aAB	896.9 aA	772.4 aAB	650.3 aB
Maltose	16.7 cdA	17.9 cdA	19.7 cdA	31.8 bA	832.6 aB
	'Cravo' mandarin				
Sucrose	0 cA	0 cA	0 Ac	0 dA	0 dA
Galactose	196.6 aA	238.0 aA	180.0 aA	226.8 aA	194.1 bA
Glucose	0 cA	0 cA	0 cA	0 cA	0 cA
Lactose	146.1 aB	276.4 aA	240.0 aA	235.0 aA	230.3 abA
Maltose	39.0 bBC	19.2 bC	61.9 bB	68.5 bB	207.3 bA
	'Itaboraí' sweet orange				
Sucrose	7.0 bA	86 cA	32.9 dA	24.6 dA	12.4 cA
Galactose	67.5 bC	330.0 aB	381.2 bB	1265.6 aA	510.8 aB
Glucose	25.8 bB	135.4 bA	152.1 cA	148.9 cA	131.8 bA
Lactose	48.2 bA	134.9 bA	45.1 cdA	36.6 cdA	29.6 bcA
Maltose	473.1 aB	389.1 aB	914.0 aA	451.4 bB	395.6 aB
	'Valencia' sweet orange				
Sucrose	0 cA	0 dA	0 dA	0 dA	0 dA
Galactose	420.8 aA	328.0 bA	428.9 bA	472.6 bA	507.9 bA
Glucose	119.9 bA	123.5 cA	122.5 cA	98.3 cA	81.2 cA
Lactose	497.3 aB	955.6 aA	893.5 aA	864.9 aA	763.7 aA
Maltose	64.4 bB	66.7 cB	157.5 cB	164.9 cB	405.5 bA
	Kinnow' m'andarin				
Sucrose	0 cA	0 cA	0 cA	0 cA	0 bA
Galactose	708.7 aAB	449.0 bB	678.4 aAB	823.9 aA	730.6 aA
Glucose	12.9 cA	28.0 cA	77.8 bA	57.7 bA	0 bA
Lactose	331.8 bB	843.8 aA	929.5 aA	901.8 aA	776.9 aA
Maltose	0 cB	0 cB	0 cB	0 cB	739.1 aA

Means followed by the same upper case letter in the horizontal level and low case letter in the vertical level do not differ among themselves (Tukey, 0.001).

Maturation and germination of somatic embryos

Our studies showed a non-significant difference among the treatments tested for the maturation of somatic embryos of 'Cravo' mandarin, 'Kinnow' mandarin and 'Itaboraí' sweet orange when the number of germinated embryos was evaluated (Table 2). However, for 'Ponkan' mandarin, a significant difference was found among the treatments. Those that had 58 and 73 mM of sucrose in combination with activated charcoal were the most effective for the maturation of 'Ponkan' somatic embryos, even though these treatments were significantly different from those that had 15 and 29 mM of sucrose in the absence of activated charcoal. The presence of activated charcoal in the culture medium did not reveal any significant difference among the tested treatments. This compound is known to adsorbing toxic compounds released to the culture medium from the explants, or from the agar, sucrose and salts. Its presence in the medium favors the growth and further development of the embryos (Debergh, 1983). Similar observations were found for the 'Valencia' sweet orange with sucrose at concentrations of 58 and 73 mM. However, these results were significant different for only those treatments that did not have sucrose. Moreover, no significant difference among the treatments with and without activated charcoal was documented.

The treatments that did not provide sucrose showed embryos with strong oxidation, even in the presence of activated charcoal. According to Ribeiro (1997), only immature embryos of *Citrus* at early stage of development require higher sucrose concentrations than 150 mM, thus allowing the embryo to reach its full development.

The presence of GA_3 in the culture medium had no effect on the maturation of embryos for all the varieties of citrus evaluated. This observation can be verified when the data collected from the treatments with GA_3 are compared to those without GA_3. Our results do not agree with those published by Button & Bornman (1971) and by Kochba et al. (1974). These authors observed that 1.0 g L^{-1} of GA_3 was enough to enhance the development of the roots of *Citrus* embryos, either partially or fully developed.

Sucrose at concentration of 44 mM associated with 0.5 g L^{-1} of activated charcoal followed by 58 mM of sucrose also with activated charcoal induced the

Table 2 - Mean number of germinated embryos and citrus plantlets produced in different treatments tested for the maturation of somatic embryos after being cultured 120 days in the germination medium, Piracicaba, SP.

MT culture medium supplemented with	'Ponkan' mandarin	'Cravo' mandarin	'Kinnow' mandarin	'Itaboraí' sweet orange	'Valencia' sweet orange
	Mean number of germinated somatic embryos per Petri dish (Number of plantlets)				
0.1 mg L^{-1} GA_3 + 73 mM of sucrose w/ activated charcoal	2.2 ab (6)	1.3 a (4)	0.7 a (0)	1.4 a (3)	1.5 ab (6)
0.1 mg L^{-1} GA_3 + 73 mM of sucrose without activated charcoal	2.2 ab (5)	1.0 a (2)	0.9 a (3)	1.3 a (4)	1.6 ab (4)
0 mM sucrose	0.7 c (0)	0.7 a (0)	0.7 a (0)	0.7 a (0)	0.7 b (0)
15 mM sucrose	1.7 b (2)	0.9 a (1)	0.7 a (0)	1.3 a (6)	1.6 ab (4)
29 mM sucrose	1.8 ab (6)	1.0 a (2)	0.9 a (2)	1.2 a (3)	1.5 ab (6)
44 mM sucrose	2.2 ab (7)	1.5 a (7)	0.9 a (3)	1.3 a (2)	1.5 ab (5)
58 mM sucrose	2.2 ab (6)	1.5 a (9)	1.2 a (4)	1.3 a (2)	1.8 a (9)
73 mM sucrose	2.2 ab (9)	1.6 a (10)	1.0 a (2)	1.3 a (2)	1.8 a (8)
0 mM sucrose w/ activated charcoal	0.7 c (0)	0.7 a (0)	0.7 a (0)	0.7 a (0)	0.7 b (0)
15 mM sucrose w/ activated charcoal	1.8 ab (6)	1.0 a (4)	1.2 a (3)	1.4 a (0)	1.5 ab (0)
29 mM sucrose w/ activated charcoal	1.7 b (7)	1.2 a (5)	1.3 a (6)	1.5 a (3)	1.6 ab (5)
44 mM sucrose w/ activated charcoal	2.3 ab (11)	1.3 a (3)	1.0 ab (2)	1.0 a (4)	1.9 a (6)
58 mM sucrose w/ activated charcoal	2.3 a (10)	1.6 a (4)	0.7 a (4)	1.5 a (2)	2.0 a (10)
73 mM sucrose w/ activated charcoal	2.3 a (9)	1.5 a (7)	0.9 a (3)	1.3 a (2)	2.1 a (15)

Means followed by the same letter in the horizontal level do not differ among themselves (Tukey, 0.001).

highest number of plantlets for 'Ponkan' mandarin. The number of plantlets produced by the different treatments is presented in Table 2. These results corroborated those of Tomaz et al. (2001), who reported that for citrus variety 'Seleta Vermelha', the highest number of plantlets was obtained in medium with 44 mM of sucrose, which was 16% greater when compared to the medium supplemented with 37 mM of maltose. Where as, 73 mM of sucrose in the absence of activated charcoal followed by 29 mM of sucrose without charcoal favored the production of the highest number of plantlets for 'Cravo' mandarin. For 'Valencia', plantlet formation occurred at 73 mM of sucrose in the presence of activated charcoal. This result does not agree with that obtained by Tomaz et al. (2001), who observed a much higher number of 'Valencia' plantlets when embryos were cultured in the presence of 44 mM of sucrose without activated charcoal. For 'Itaboraí', the highest number of plantlets was obtained at 15 mM of sucrose without activated charcoal, while for 'Kinnow', plantlet formation occurred at 58 mM of sucrose associated to activated charcoal.

Sucrose was an important carbon source for embryo germination and the formation of plantlets for all the varieties of citrus analyzed. Somatic embryos that were initially cultured at 44 mM of sucrose plus charcoal ('Ponkan' mandarin), at 73 mM of sucrose minus charcoal ('Cravo' mandarin), at 29 mM of sucrose plus charcoal ('Kinnow' mandarin), at 15 mM of sucrose minus charcoal ('Itaboraí' sweet orange), and 73 mM of sucrose plus charcoal ('Valencia' sweet orange), and subsequently transferred to the germination medium formed plantlets at the rate of 73%, 66%, 40%, 40% and 100%, respectively (Table 2).

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