Review Article

การเพาะเลี้ยงเนื้อเยื่อพืชสมุนไพรมะระขี้นกและการประยุกต์ใช้

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บทคัดย่อ

การเพาะเลี้ยงเนื้อเยื่อพืชสมุนไพรมะระขี้นกและการประยุกต์ใช้
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มะระขี้นก (*Momordica charantia* L.) เป็นพืชในวงศ์ Cucurbitaceae ซึ่งใช้บริโภคเป็นผักสมุนไพรและใช้ในการแพทย์แผนโบราณและการแพทย์พื้นบ้านต่างๆ มาเป็นเวลานาน มีรายงานการศึกษาเกี่ยวกับฤทธิ์ทางเภสัชวิทยาของมะระขี้นกที่หลากหลาย เช่น ฤทธิ์ลดน้ำตาลในเลือด ฤทธิ์การปรับภูมิคุ้มกัน ฤทธิ์ต้านเชื้อจุลชีพ และฤทธิ์ต้านออกซิเดชัน สารสำคัญในมะระขี้นกประกอบด้วยสารกลุ่มต่างๆ ได้แก่ ไตรเทอร์พีนอยด์ ฟลาโวนอยด์ กรดฟีนอลิก โพลีแซคคาไรด์ เปปไทด์ และโปรตีน บทความนี้ได้รวบรวมรายงานการวิจัยเกี่ยวกับการใช้กระบวนการทางชีวภาพเพื่อการเพาะเลี้ยงและเพิ่มปริมาณสารสำคัญในต้นมะระขี้นก วิเคราะห์และอภิปรายการใช้สารควบคุมการเจริญของพืชและปัจจัยต่างๆที่ส่งผลกับการเพื่อกระตุ้นให้เกิดยอดโดยตรง การกระตุ้นให้เกิดยอดผ่านการชักนำแคลลัส และการกระตุ้นให้เกิดยอดผ่านการชักนำโซมาติกเอมบริโอ รวมถึงการชักนำให้เกิดรากลอยเพาะเลี้ยงด้วยแบคทีเรีย *Agrobacterium rhizogenes* และปัจจัยที่มีผลกับปริมาณสารสำคัญในเนื้อเยื่อเพาะเลี้ยงของต้นมะระขี้นก

คำสำคัญ: มะระขี้นก, เพาะเลี้ยงเนื้อเยื่อ, รากลอย, การขยายพันธุ์, สารควบคุมการเจริญเติบโต

In Vitro Cultures of Medicinal Plant *Momordica charantia* L. and Their Applications

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Abstract

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Momordica charantia L. (Cucurbitaceae), a medicinal plant that has been used in folk medicines for centuries, reportedly possesses a wide range of biological activities such as hypoglycemic, immunomodulatory, antimicrobial, and antioxidant activities. Various bioactive groups of compounds including triterpenoids, flavonoids, phenolic acids, polysaccharides, peptides and proteins from *M. charantia* have been reported. Different biotechnological approaches have been used as alternative to cultivate *M. charantia* and in attempt to increase its bioactive compounds. Protocols for cultivation of *M. charantia* from *in vitro* direct and indirect organogenesis are summarized in this review. Transformation of *Agrobacterium rhizogenes* to generate hairy root cultures of *M. charantia* is reported. The influences of factors affecting different plant tissue formations and bioactive compounds accumulated in *in vitro* cultures are reviewed and discussed.

Keywords: *Momordica charantia*, tissue culture, micropropagation, hairy root, plant growth regulators

Introduction

Momordica charantia L., commonly known as bitter gourd, bitter melon, or balsam pear, is a popular vegetable and a well-known medicinal plant of the family Cucurbitaceae. The plant is native to tropical and subtropical parts of the world. It is widely distributed in many countries including Thailand, Malaysia, Vietnam, Singapore, India, China, Japan, Brazil, Cuba, Ghana, Haiti, Mexico, East Africa, Middle East, Central and South America (Jia *et al*., 2017).

Despite the fruit and other parts of the plant taste very bitter as suggested in its common name, bitter gourd has been used as culinary vegetable for long time. It has also been used as traditional herbal medicines as well in many countries. In Ayurvedic medicines, *M. charantia* is used for treatment of diabetes, diarrhea, pain and fever (Grover *et al*., 2014). In Turkish folk medicines, its mature fruits are used externally for treatment of wounds. The plant is also used for gynecological aid, fevers, and diabetes by traditional healers in Togo (Beloin *et al*., 2005).

M. charantia has attracted great interest in recent years as it is a high nutritional vegetable and also a medicinal plant. Several studies have reported their pharmacological activities including antidiabetic, anticancer, hypotensive, anti-obesity, antimicrobial, antihyperlipidemic, antioxidant, anti-inflammatory, immunomodulatory, anthelmintic, neuro-protective and hepato-protective both *in vitro* and *in vivo* (Grover *et al*., 2004; Jia *et al*., 2017; Telang *et al*., 2003; Wang *et al*., 2017; Zhang *et al*., 2018). Currently, various health products from *M. charantia* such as tea infusion, capsulated extracts, and other forms of dietary supplements, are popularly available worldwide.

Biotechnological approaches play important role in improvement of quality of raw materials and enhancement of bioactive compounds production. *In vitro* plant propagation by plant cell and tissue culture technology is an alternative platform to effectively and rapidly produce large number of consistent plant materials. Application of different techniques such as use of plant growth regulators or elicitors with *in vitro* plant cultures under controlled conditions can further lead to higher production of plant bioactive secondary metabolites. Increasing number of successful *in vitro* culture protocols have been established and reported in many medicinal plant species. The present review summarizes the achievements of biotechnological techniques for micropropagation and production of secondary metabolites in *M. charantia.*

Chemical compositions and biological activities

M. charantia contains diverse groups of phytochemicals including triterpenoids, phenolic acids, flavonoids, polysaccharides, proteins and other functional components. Several studies have reported various bioactive groups of compounds found in different parts of this plant (Table 1). Two classes of saponins recognized as cucurbitane- and oleanane- type triterpenoids are presented in *M. charantia*. More than 50 cucurbitane-type triterpenoids have been isolated from different parts of the plant (Wang *et al.*, 2017). Cucurbitacins are a group of bitter compounds associated with antidiabetic and hypoglycemic activities (Chen *et al,* 2005). Charantin, which is an equal mixture of β-sitosteryl glucoside and 5,25-stigmasteryl glucoside, is a natural cucurbitane type triterpenoid that can be isolated from fruit, leaves, and seeds of *M. charantia* (Cuong *et al,* 2017). Flavonoids and phenolic compounds are also presented in fruit and other parts of this plant. Phenolic compounds including gallic acid, catechin, quinic acid, epicatechin, chlorogenic acid, coumaric acid, benzoic acid, ferulic acid, cinnamic acid, and sinapinic acid were distributed in various amount among each part of different plant tissues (Horax *et al.*, 2005). Extracts of *M. charantia* containing these phenolic compounds have been reported to exhibit antioxidant activity (Ibrahim *et al.*, 2010). Polysaccharides which have been identified as one of the main hypoglycemic components in *M. charantia* (Raish *et al.*, 2016; Zhang *et al.*, 2018) are presented in various parts of the plant. Polysaccharides extracted from *M. charantia* exhibited a wide range of biological activities, such as immunomodulatory, neuroprotective, anti- ulcer and antioxidant activity (Deng *et al,* 2014; Gong *et al.*, 2015; Panda *et al.*, 2015; Tan and Gan, 2016). Proteins and peptides are also rich in fruits and seeds of *M. charantia.* These proteins and peptides including *M. charantia* cyclic peptides, *M. charantia* polypeptide MC6, ribosome activating proteins (RIPs), *M. charantia* lectin (MCL), Momordica anti-HIV protein of 30 kD (MAP30), α-momorcharin (α-MMC) and β-momorcharin (β-MMC) have been reported to potentially possess bioactivities such as hypoglycemic, anti-tumor and antimicrobial activities (Jia *et al.*, 2017; Mahatmanto *et al.,* 2015; Wang *et al.,* 2012).

Biotechnology approaches for *M. charantia in vitro* propagation

Biotechnological approaches are useful for multiplication of plant materials and enhancement of bioactive compounds production. Micropropagation is the alternative tool to rapidly multiply plant materials. *In vitro* propagation protocols for *M. charantia* have been reported by several groups of researchers using Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with various plant growth regulators (Table 2). Various factors have been reported to affect the micropropagation such as genotype of the plant, explant types, and the concentration and combination of different components of the culture media.

Table 1 Bioactive components of *M. charantia*

Major Bioactive components	Compounds	Distribution	References
Triterpenoids	Charantin Cucurbitacins Momordicosides	Fruit, leaf, seed, stem, root	Chen *et al*., 2005 Cuong *et al*., 2017 Wang *et al*., 2017 Xu and Dong, 2005
Flavonoids and phenolic acids	Gallic acid Catechin Quinic acid Epicatechin Coumaric acid Benzoic acid Cinnamic acid etc.	Fruit, seed	Horax *et al*., 2005
Polysaccharides	*M. charantia* Polysaccharides BP1-3 Polysaccharides MCPs etc.	Fruit, seed, leaf, root	Zhang *et al*., 2016
Protein and peptides	*M. charantia* polypeptide MC6 *M. charantia* peptide Lectin α-momorcharin β-momorcharin etc.	Fruit, seed	Jia *et al*., 2017 Wang *et al*., 2012

Table 2 Summary of *M. Charantia in vitro* cultures

Type of culture	Optimal culture conditions	Explants used	Response	Reference
Shoot multiplication (Direct organogenesis)	MS + 0.5 mg/L BAP	Shoot tip, nodal, and internodal explants from *in vitro* seedlings	4.0-4.9 shoots/explant	Agarwal and Kamal, 2004
Shoot multiplication (Direct organogenesis)	MS + 1.0 mg/L BAP + 0.1 mg/L TDZ	Shoot tip from *in vitro* seedlings	2.75 shoots/explant	Malik *et al*., 2007
Callus induction	MS + 1.0 mg/L BAP or 1.5 mg/L NAA or 1.0 mg/L TDZ	Leaf, stem, and cotyledon explants from *in vitro* seedlings	100% callus response but no further regeneration was observed	Malik *et al*., 2007
Callus induction	MS + 1.0 mg/L 2,4-D + 2.0 mg/L BAP	Stem explants	78.3% callus induction but no further regeneration	Tang *et al*., 2011

Table 2 Summary of *M. Charantia in vitro* cultures (*Continue*)

Type of culture	Optimal culture conditions	Explants used	Response	Reference
Shoot regeneration (Indirect organogenesis)	MS + B5 vitamins + 1.2 mg/L TDZ + 0.4 mg/L NAA + 0.6 mg/L $AgNO_3$	Callus from leaf explants	30-40 shoots/explant	Thiruvengadam *et al.*, 2010
Callus induction	MS + B5 vitamins + 0.5 mg/LTDZ + 1.6 mg/L NAA	Immature leaf from *in vitro* seedlings	94.4% callus response	
Somatic embryogenesis (Indirect organogenesis)	Liquid MS + 1.5 mg/L 2,4-D	Cell suspension cultures from leaf derived explants	24.6% globular embryo regeneration	Thiruvengadam *et al.*, 2006
Callus induction	MS + 1.0 mg/L 2,4-D	Leaf explants from *in vitro* seedlings	90% callus response	
Hairy root induction	Liquid MS + 250 mg/L cefotaxime sodium salt	Cotyledon and leaf explants from *in vitro* seedlings	25-37.5% transformation frequency	Swarna and Ravindhran, 2012
Hairy root induction	Liquid MS	Leaf explants from *in vitro* seedlings	91.5% transformation frequency	Thiruvengadam *et al.*, 2014

Abbreviations:

2,4-D	2,4-dichlorophenoxyacetic acid
B5	Gamborg B5 medium (Gamborg *et al*., 1968)
BAP	6-benzylaminopurine
MS	Murashige and Skoog medium (Murashige and Skoog, 1962)
NAA	1-naphthaleneacetic acid
TDZ	thidiazuron

Direct organogenesis by shoot induction

Direct organogenesis by shoot induction provides simple and reliable method for micropropagation. High rate of plant materials multiplication with low clonal variation can be obtained by subcultures of small shoots induced from each explant. *In vitro* plant regeneration of *M. charantia* through direct shoot formation was reported (Agarwal and Kamal, 2004). Shoot apex, nodal and internodal explants from *in vitro* seedlings were cultured on MS medium supplemented with different cytokinin plant growth regulators (0.0-6.0 mg/L 6-benzylaminopurine (BAP) or kinetin (KN)) for multiple shoots induction. Shoots were observed after 20 days of culture. Highest shoot production (4.0-4.9 shoots per explant) was obtained from all types of explants cultured on MS medium supplemented with low concentration (0.5 mg/L) of BAP. After shoot multiplication, root induction was carried out on MS medium supplemented with different concentration of auxins (0.0-5.0 mg/L indole-3-acetic acid (IAA, indole-3-butyric acid (IBA), or 1-naphthaleneacetic acid (NAA)). Root formation was observed after 22 days of culture. Best response was obtained from MS medium supplemented with 3.0 mg/L IBA from shoot tip and internodal explants. In this study, the plantlets were successfully transferred to culture in pots in green house with 40% plant survival rate. Another study investigated *in vitro* plant regeneration via direct organogenesis focused on effect of single and combination of various plant growth regulators (Malik *et al*., 2007). Highest shooting response (2.75 shoots per explant) was obtained from MS medium supplemented with 1.0 mg/L BAP in combination with 0.1 mg/L thidiazuron (TDZ) for shoot tip explants and 1.5 mg/L BAP in combination with 0.2 mg/L NAA for cotyledon node explants. Root formation was also succeeded from shoots transferred to MS medium supplemented with different concentrations of auxins.

Indirect organogenesis via callus induction

Indirect organogenesis involves the regeneration of shoots from morphogenic callus. Unlike direct organogenesis, shoot induction via intervening callus stage is often associated with somaclonal variation. However, this variability can be advantage for development or selection of good characteristic cultivar. For indirect organogenesis of *M. charantia*, leaf, stem and cotyledon explants were used for induction of callus cultures on MS medium supplemented with various concentration of different auxin and cytokinin (0.0 - 3.0 mg/L of 2,4-D, BAP, NAA, and KIN). Successful callus induction was observed from all types of explants. The callus character was compact and green. However, indirect organogenesis by further induction of shoot from these calluses was not success (Malik *et al*., 2007).

Another study reported the successful indirect organogenesis from callus-derived leaf explants of *M. charantia* by using of TDZ, NAA, and silver nitrate ($AgNO_3$) (Thiruvengadam *et al*., 2010). Leaves from *in vitro* seedlings and mature leaves from plant growing in a plant chamber were used as explants for callus induction. For experiment carried out on MS medium supplemented with B5 vitamins and different individual auxins, 2,4-D yielded yellowish friable callus, NAA yielded greenish friable callus, while IAA yielded brownish friable callus, but none of the calluses induced by single auxin was succeeded in further organogenesis. However, when combination of auxins (0.0-8.8 µM of 2,4-D or NAA or IAA) and cytokinins (0.0-4.5 µM of BAP or TDZ) were used, nodular greenish compact callus was obtained. For adventitious shoot formation, callus induced from MS medium supplemented with 7.7 µM NAA and 2.2 µM TDZ which showed best callus response was cultured for 3 weeks before transferred to MS medium supplemented with different concentrations of TDZ, NAA, and $AgNO_3$. Regeneration of 30-40 shoots per explant was achieved on MS medium supplemented with 5.5 uM TDZ, 2.2 µM NAA, and 3.3 µM $AgNO_3$. The shoots of around 1.0 cm length were elongated in MS medium containing 3.5 µM gibberellic acid and rooted in MS medium containing 4.0 µM IBA. Rooted regenerated plants were acclimatized in the green house and finally in soil with 90% survival rate. This study established a protocol for micropropagate 40 plants per leaf explant in a culture period of 98 days.

Indirect organogenesis via callus induction is known to be influenced by concentration of endogenous and exogenous auxins and cytokinins. Correlation between endogenous hormones and the formation of buds from callus induced by placing stems explant of *M. charantia* on MS medium supplemented with 1.0 mg/L 2,4-D and 2.0 mg/L BAP was investigated. It was reported that the endogenous zeatin (ZT) was higher in the callus that showed bud formation compared with IAA, abscisic acid (ABA) and gibberellins 3 (GA3). However, although there was successful bud formation from callus induced by 2,4-D and BAP, no further regeneration was reported (Tang *et al*., 2011).

Indirect organogenesis via somatic embryogenesis

Somatic embryogenesis is the process which embryo-like structures are formed and regenerated to a complete plant. Indirect organogenesis via somatic embryogenesis has benefits over conventional propagation as it allows simultaneous production of root and shoot and also is capable of rapid large scale production. Indirect organogenesis via somatic embryogenesis from suspension culture of *M. charantia* was reported by Thiruvengadam *et al.* (2006). Suspension cultures obtained by growing calluses of *M. charantia* in liquid MS medium containing 0.0-2.0 mg/L 2,4-D showed rapid cell division of calluses. High frequency of globular embryos (24.6%) was formed in suspension culture from MS medium supplemented with 1.5

mg/L 2,4-D. These embryos differentiated into heart and torpedo stages within 2 weeks after complete removal of 2,4-D from the culture media in further subcultures. They reported successfully germination of somatic embryos on solidified MS medium with no additional growth regulators. The authors also investigated effects of components of culture media and stimulating factors such as carbohydrates and amino acids on formation of somatic embryos. They reported that full strength MS media supplemented with 50 mg/L polyvinylpyrrolidone (PVP) and 40 mg/L glutamine was effective to achieve a high frequency of somatic embryo induction, maturation and further development.

Exogenous polyamines are known to induce cell division and increase regeneration in plant cell cultures including somatic embryogenesis (Baron and Stasolla, 2008; Kakkar *et al.*, 2000). Role of polyamines during induction of somatic embryos in many plant species has been reported (Kumar *et al.*, 2008; Rajesh *et al.*, 2014; Wu *et al.*, 2009). After Thiruvengadam *et al.* (2006) successfully optimized somatic embryogenesis in *M. charantia*, another study has investigated the effects of polyamines on somatic embryogenesis in this plant (Paul *et al*, 2009). Scanning electron microscopy was used to determine somatic embryogenesis in leaf explants induced by MS medium supplemented with 0.5 mg/L NAA and 5 mg/L BAP. Embryogenic calluses showed globular embryos, heart shaped embryos, torpedo shaped embryos, and cotyledonary staged embryos from 15 to 35 days of culture. The results of endogenous free polyamines monitored during 42 days of culture content showed higher titers of putrescine than spermidine and spermine. When adding exogenous polyamines to the embryogenic media, increases in fresh weights of embryogenic calluses and number of somatic embryos were observed. Maximum increase in fresh weights at 5.0 fold and maximum increase in number of somatic embryo production at 2.5 fold were obtained from addition of 1 mM exogenous putrescine. These results showed the role of polyamine putrescine as one factor that could promote induction of somatic embryos.

Hairy root cultures

Hairy root culture, also known as transformed root culture, is a biotechnological approach utilizing the infection of wounded plant tissues with *Agrobacterium rhizogenes* to yield hairy root lines which often possess comparable production of bioactive compounds to native plant roots. Due to its advantages of high stability and productivity characters, the exploitation of hairy roots has become a valuable tool for production of medicinal plant biomass and bioactive compounds. Moreover, these hairy root cultures can also be further elicited to enhance bioactive compound accumulation in both small and large scale production. A number of studies published successful protocols to increase secondary metabolite production in many medicinal plant species.

Hairy root culture of *M. charantia* was achieved by infection of 10-day-old cotyledon and leaf explants with *A. rhizogenes* strain MTCC 532. Roots emerged from wounded explants were observed from 10 days after complete removal of bacteria by cultivation on MS medium supplemented with 250 mg/L cefotaxime. However, root emergence frequency was low, 37.5% and 25% for leaf and cotyledon explants, respectively (Swarna and Ravindhran, 2012).

Other study has reported hairy root induction of two cultivars of *M. charantia* using leaf, cotyledon, hypocotyl, root, and node explants by infection of *A. rhizogenes* strain KCTC 2703 and KCTC 2704. Roots formation could be observed from 2-5 days after inoculation of *A. rhizogenes* and hairy root characters were developed within 20 days of inoculation. Best condition for hairy root induction was obtained from infection of *A. rhizogenes* strain KCTC 2703 on leaf explants of *M. charantia* both cultivar Korean and Indian with 85.2±1.0% and 91.5±1.2% induction, respectively. Moreover, as different components of culture medium can significantly affect growth of *in vitro* plant cultures (Rao and Ravishankar, 2002), the authors also investigated effects of different culture media including MS medium, Gamborg's B5 (B5) medium (Gamborg *et al.*, 1968), Nitsch and Nitsch (NN) medium (Nitsch and Nitsch, 1969), and Chu's (N6) medium (Chu, 1978) on growth of hairy root cultures. Results from optimization of culture conditions suggested that basal liquid MS medium yield the highest biomass of hairy roots when compared with other media which have different compositions of macro- and micro- nutrients (Thiruvengadam *et al.,* 2014).

Secondary metabolites in *in vitro* cultures

Plant cell and organ culture play important role in production of natural bioactive compounds from plants. *In vitro* cultures for secondary metabolite production include unorganised tissue (callus and cell suspension cultures) and organised tissue (shoot, root, whole plant, and transformed roots) cultures. Production of plant secondary metabolites in these vitro cultures could be enhanced by application of advance techniques such as elicitation, precursor feeding, and cell immobilization (Guerriero *et al*, 2018; Isah *et al*., 2018; Rao and Ravishankar, 2002). Although a large number of publications related to accumulation of chemical compounds in *M. charantia* and their bioactivities have been reported, studies of bioactive compounds from *in vitro* plant cell and tissue cultures are limited.

Swarna and Ravindhran (2012) reported detection of charantin, a cucurbitane type triterpenoid with potential hypoglycemic activity, in hairy root culture of *M. charantia* by thin layer chromatography. The authors compared chromatographic results from methanolic extracts of hairy roots, leaf, and fruit by expected retention factor value of charantin. However, no quantitative analysis was reported.

Production of phenolic compounds in hairy root cultures of *M. charantia* was studied (Thiruvengadam *et al*., 2014). Qualitative and quantitative determination of phenolic compounds in hairy roots of Indian and Korean cultivars of *M. charantia* were studied in comparison with results from untransformed roots. A total of 27 phenolic compounds were identified from both transformed and untransformed roots. Hairy root culture of *M. charantia* exhibited higher total phenolic and flavonoid compound accumulation and also superior antioxidant and antimicrobial activity than in untransformed roots.

Other study focused on application of elicitors to enhanced the production of phenolic compounds and their biological activities in hairy root cultures (Chung *et al*., 2016). The results showed jasmonic acid and salicylic acid significantly increase production of total phenolic and flavonoid contents in elicited hairy roots (4.1 and 3.5 mg/g, respectively) than in non-elicited hairy roots (3.7 and 3.2 mg/g, respectively). They also reported higher bioactivities of hairy root cultures in antioxidant activity by DPPH assay, antimicrobial activity, and anticancer activity by MTT assay.

Previous studies reported various *in vitro* cultures of *M. charantia* which are effective protocols for propagation and production of plant biomass as medicinal plant raw materials. However, few studies have reported successful enhancement of bioactive compounds in *in vitro* cultures of *M. charantia* to date. Advances in biotechnological techniques to modify *in vitro* plant cultures such as cell suspension culture, immobilization, and application of plant growth regulators, elicitors, or precursors of plant bioactive compounds which are repeatedly reported to enhance production of secondary metabolites in many medicinal plant species could also further improve accumulation of bioactive compounds in *in vitro* cultures of *M. charantia*.

Conclusion

M. charantia has gained increasing interests in recent years due to its functional components and potential health benefits. To date, several biotechnological protocols for cultivation and enhancement of secondary metabolites have been reported. Plant growth regulators play important role in organogenesis of *in vitro* cultures. Development of transformed hairy root culture could be used to increase production of phenolic and flavonoid contents. However, studies related to secondary metabolite production in callus and cell suspension cultures are still lacking. Moreover, detailed metabolic profiles and bioactivity assessment in *in vitro* cultures are the challenging opportunity to explore which could be beneficial for improvement of raw material of *M. charantia* for further production in larger scales.

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