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# Plant regeneration from various explants of cultivated *Piper* species

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**Abstract.** Morphogenetic potential of root, leaf, node and internode explants of 3 cultivated *Piper* species was investigated to develop a reliable plant regeneration protocol. *P. longum* (pipli) was the most responsive followed by *P. betle* (betel vine) and *P. nigrum* (black pepper). In *P. longum* the highest number of shoot buds was produced on root explants followed by node, internode and leaf explants. In *P. betle* and *P. nigrum* adventitious shoot buds differentiated only from internodal and nodal ring regions, respectively. Histological examination in *P. longum* showed that adventitious shoot buds originate directly from the cortical cells of the root and the internode without an intervening callus phase. Benzyladenine was superior to kinetin for shoot induction and its optimum concentrations for *P. longum, P. betle* and *P. nigrum* were 1-2, 10 and 10 μM, respectively. Shoot elongation and rooting were achieved in $B_5$ medium containing 0.5 μM benzyladenine and 1 μM indoleacetic acid, respectively. Regenerated plants were established in soil.

**Abbreviations:** BA: $N^6$ benzyladenine; $B_5$ medium: Gamborg et al. (1968) medium; IAA: indole-3-acetic acid.

## Introduction

The genus *Piper* has over 1000 species of which the 3 important cultivated species are *P. nigrum* L. (black pepper), *P. betle* L. (betel vine) and *P. longum* L. (pipli), in that order. Cultivation of *P. longum* is restricted to India and its spikes are used in pickles, medicine and Indian cooking. Leaves of *P. betle* are chewed with betelnut (*Areca catechu* L.) in many countries from Tanzania (Zangibar Islands) through South and Southeast Asia and into the Pacific (The Wealth of India 1969). *P. nigrum* is highly valued all over the world for its unique flavour and is routinely used in seasoning food and curing meat.

In *Piper* both dioecious and hermaphrodite forms occur, and cultivated forms are clonally propagated through cuttings. In black pepper hermaphrodite forms are preferred, while most of the betel vine cultivars grown in India are male (The Wealth of India 1969). A number of factors such as high heterozygosity, polyploidy, dioecious nature etc. hamper breeding improvement of *Piper* species. So far only one cultivar of black pepper, Panniyur 1, has been released for cultivation in India.

Tissue culture techniques can play an important role in clonal propagation, germplasm conservation and plant improvement of *Piper* species. Limited work on tissue culture of *Piper* has shown that *in vitro* establishment is severely hampered by high incidence of bacterial contamination (Mathews & Rao 1984, Fitchet 1990). Recently micropropagation of black pepper (Philip *et al.* 1992) and regeneration of plants from callus cultures of *P. longum* (Bhat *et al.* 1992) and *P. betle* (Aminuddin *et al.* 1993) have been reported. With the objective of employing tissue culture techniques for genetic manipulation and somaclonal improvement, we examined plant regeneration potential of three cultivated *Piper* species from various explants.

## Materials and methods

*Establishment of aseptic cultures. In vitro* cultures of *P. nigrum, P. betle* and *P. longum* were initiated from terminal portions of the vines raised in pots. Cuttings bearing 4-5 nodes were thoroughly washed in detergent water and swabbed with cotton wool dipped in rectified spirit. Explants were surface

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sterilized in 0.1% mercuric chloride solution for 10-12 min followed by at least 6 rinses in sterile distilled water. Single nodal pieces were prepared and inoculated vertically in semisolid Murashige and Skoog (1962) medium containing 3% sucrose and 1 μM BA. Cultures were incubated at 25 ± 2°C, 16 h photoperiod (30 μmol $m^{-2}$ $s^{-1}$) provoded by cool white fluorescent lamps. After discarding contaminated cultures, surface sterilization was repeated during first 2 subcultures, and healthy cultures were multiplied by subculture at 8 week intervals on $B_5$ medium (Gamborg *et al.* 1968) with 1 μM BA. All media contained 0.8% agar, pH was adjusted to 5.8, were dispensed into tubes and autoclaved for 15 min at 121°C.

*Shoot bud induction and plant regeneration.* Shoot regeneration potential of 4 types of explants, root, leaf, internode and node, was assessed on $B_5$ medium supplemented with BA or kinetin (1, 2, 5 and 10 μM). Root explants (2-3 cm) were taken from cultures raised on $B_5$ medium supplemented with 1 μM IAA while other explants (1-2.5 cm size) were derived from shoot cultures growing on $B_5$ medium containing 1 μM BA. Nodal explants were placed vertically with the nodal ring in contact with the medium whereas other explants were placed horizontally on the medium. Explants with induced shoot buds were transferred to $B_5$ medium containing 0.5 μM BA for shoot growth, and later rooted on $B_5$ medium with 1 μM IAA. Each treatment was replicated at least 12 times and the experiment was repeated twice. Observations on shoot bud induction were recorded after 6 weeks.

*Hardening and establishment in soil.* Four-week-old *in vitro* regenerated plants were washed in tap water and transplanted into pots filled with sterilized 'Soil rite'. Pots were covered with polythene bags to provide high humidity around plants and kept in shade in a nethouse. Polythene covers were gradually removed over a period of 2 weeks, and after 4 weeks plants were transferred to soil.

*Histology.* Explants were collected after various periods of culture and fixed in formalin:acetic acid:ethanol (1:1:18) for 48 h. Material was dehydrated by passing through a graded series of t-butanol and embedded in paraffin wax. Serial sections of 6-8 um thickness were cut and stained with 1% Toluidine blue O in 50% ethanol and examined under a binocular microscope.

## Results and Discussion

Establishment of *in vitro* cultures of *Piper* species was slow and difficult. Bacterial contaminations were observed in more than 90% of primary cultures. Even apparently healthy cultures showed bacterial growth following subculture. However, by repeating surface sterilization of newly emerging shoots during the first 2 subcultures, healthy cultures could be established in all 3 species.

Endogenous bacterial contamination causing severe setback to *in vitro* establishment of *P. nigrum* has been reported by several workers (Mathews and Rao 1984, Fitchet 1990, Philip *et al.* 1992). Repeated surface sterilization has been reported to delay the onset of bacterial growth but not to eliminate them (Fitchet 1990, Philip *et al.* 1992). Our success may be attributed to careful excision and surface sterilization of newly emerging shoots, rather than repeating the surface sterilization on the original explants as done by previous workers. Perhaps the titre of slow-growing bacteria is reduced and ultimately eliminated during successive surface sterilizations.

Morphogenic potential of *P. longum* explants in $B_5$ medium supplemented with various concentrations of BA or kinetin is summarised in Table 1. All explants produced some callus at the cut ends in almost all treatments. Number of shoot buds induced varied greatly between treatments and their counting was difficult in some cases. Hence, instead of the average, the range of shoot buds induced per explant in each treatment is presented.

Among the 4 types of explants, root was the most responsive and gave rise to numerous shoot buds from all over the explant (Fig. 1A). Shoot buds regenerated from the surface of the leaf explants (Fig. 1B) whereas internode explants produced shoot primordia at the cut ends (Fig. 1C). Besides axillary shoots, nodal explants regenerated adventitious shoot buds from the proximal end of the internode and also around the nodal ring (Fig. 1D). Maximum number of shoot bud differentiation at the nodal ring was observed at low concentrations (1-2 μM) of BA, whereas higher concentrations led to the formation of soft, friable callus. The response at the proximal end of the node was similar to the internode explants, and hence in the table shoot buds originating at the nodal ring only are given. Shoot bud differentiation was recorded in 100 per cent of the induced explants.

BA was more effective than kinetin for *de novo* shoot bud formation (Table 1). For all explants except the internode, the highest number of shoot buds was produced at 1-2 μM BA; higher concentrations suppressed shoot regeneration and gave rise to green, nodular callus. The internode explants gave the best response at 10 μM kinetin. In kinetin-supplemented media internode explants regenerated roots as well as shoots. Kinetin, however, failed to induce shoot buds on leaf explants and was less effective than BA for shoot regeneration on root and node explants.

Root and leaf explants of *P. betle* and *P. nigrum* produced callus but failed to show organogenesis. Callus of betel vine was soft and friable whereas that of black pepper was spongy and white. In *P. betle* shoot bud regeneration was observed on internode and nodal explants (Table 2). However, nodal explants produced adventitious shoot buds from only the proximal end of the internode (Fig. 2A); nodal ring tissue gave rise to soft, friable callus. In contrast to *P. betle*, internodal region of *P. nigrum* failed to produce shoot primordia while adventitious regeneration was obtained from the

**Table 1.** Response of *P. longum* explants to varying cytokinin levels in $B_5$ medium containing 3% sucrose.

| Cytokinin | E X P L A N T | | | |
|---|---|---|---|---|
| (μM) | Root | Leaf | Internode | Node |
| 1 BA | C, S (>50)[a] | C, S (10-15) | C, S (15-20) | R, S (>30) |
| 2 BA | C, S (>50) | C, S (10-15) | C, S (15-20) | R, S (>30) |
| 5 BA | C, S (4-5) | C, S (1-2) | C, S (5-7) | C |
| 10 BA | C | C | C, S (3-5) | C |
| 1 Kinetin | C, S (10-15) | C | R, C | R, C |
| 2 Kinetin | C, S (10-25) | C | R, C, S (1-2) | R, C |
| 5 Kinetin | C, S (>30) | C | R, C, S (5-7) | C, S (2-5) |
| 10 Kinetin | S (>30) | C | R, C, S (20-25) | C, S (5-10) |

a: C-Callus, R-Root, S-Shoot bud. Figures in parentheses indicate the number of shoot buds formed per explant.

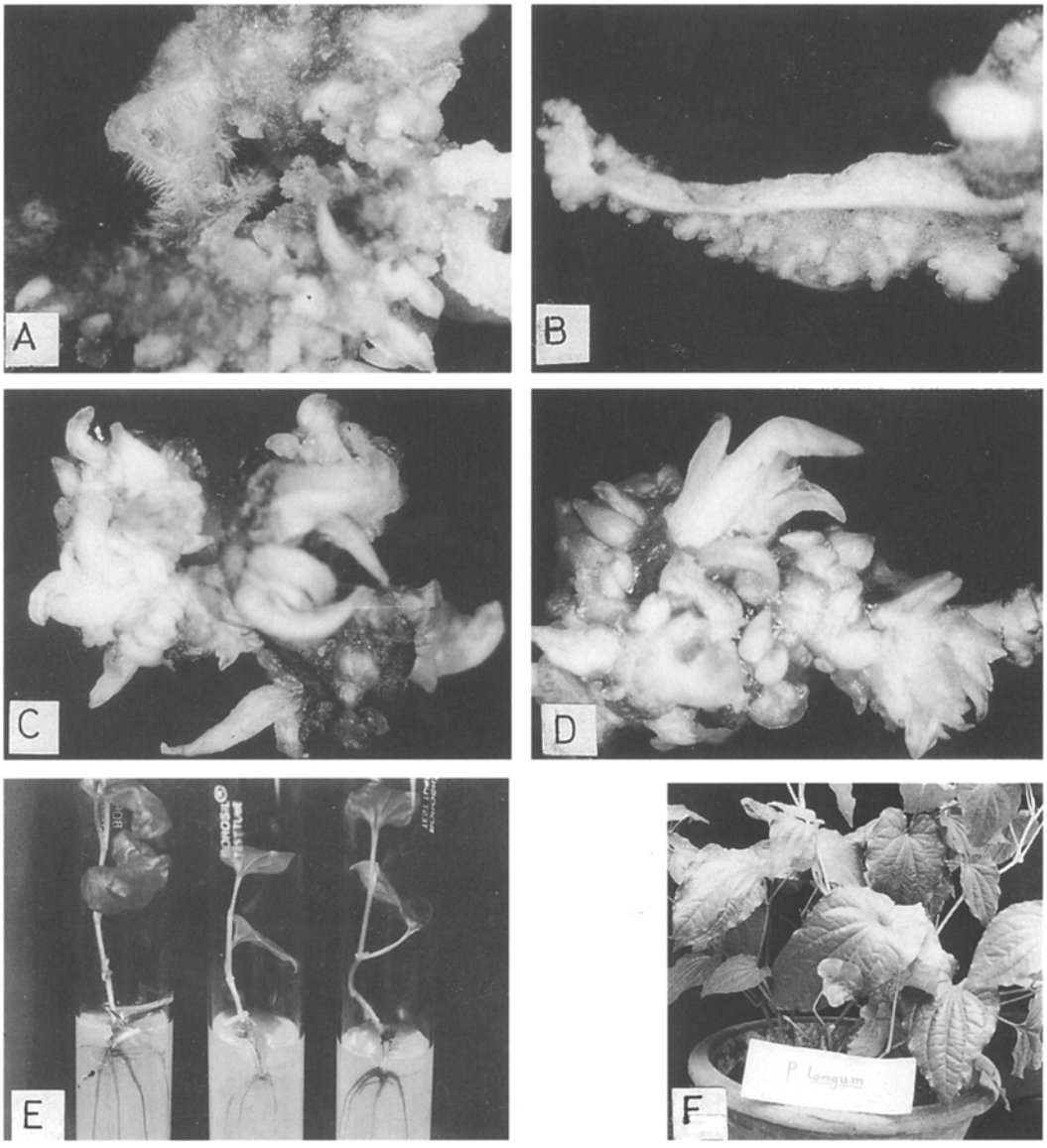


**Fig. 1.** Plant regeneration from various explants of *P. longum*. A-D, Shoot bud differentiation from root (A), leaf (B), internode (C) and node (D) explants. E, Rooting of shoots in $B_5$ medium with 1 uM IAA. F, Regenerated plants established in soil.

nodal ring tissue (Fig. 2D, Table 2). About 10 adventitious buds were produced per explant around the nodal ring.

As with *P. longum*, BA was better than kinetin for shoot bud regeneration in betel vine and black pepper. A higher concentration of BA (5-10 μM) was necessary to elicit good response. However, even at the best concentration only 40% of the nodal explants of *P. nigrum* responded with adventitious shoot buds. Kinetin failed to induce shoot buds in *Piper nigrum* but supported axillary bud proliferation. Our results are in agreement with that of Philip *et al.* (1992), who found 13.6 μM BA to be optimum for micropropagation of *P. nigrum*.

Shoot buds induced using 5-10 μM BA had to be transferred to a medium containing 0.5 μM BA for further development into shoots. After 4-6 weeks, 1-3 cm shoots were produced in all species. All shoots regenerated 2-4 roots within 2 weeks of transfer to a medium containing 1 μM IAA (Figs. 1E, 2B, 2E). Such regenerated plants were successfully established in soil (Figs. 1F, 2C, 2F).

Histological examination of adventitious shoot regeneration was carried out in *P. longum*. Roots of *P. longum* show typical dicot anatomy with a single-layered epidermis, multilayered cortex, endodermis, xylem and phloem (Fig. 3A). Mitotic activity in cultured roots was first observed in cortical cells, which ultimately led to disruption of the epidermis (Fig. 3B). Such meristematic activity was seen at several places and in different layers of the cortex. The meristematic regions, on further division, formed a notch-like structure (Fig. 3C) which ultimately gave rise to shoot buds. Regeneration from the internode explants also followed a similar pattern and, as in the root, cortical cells were involved in shoot bud differentiation (Fig. 3D). Some loose callus was produced as a result of disruption of the epidermis, but it did not produce shoot buds.

**Table 2.** Response of *P. betle* and *P. nigrum* explants to varying cytokinin levels in $B_5$ medium containing 3% sucrose.

| Cytokinin (μM) | *P. betle* | | *P. nigrum* | |
|---|---|---|---|---|
| | Internode | Node | Internode | Node |
| 1 BA | 1-2 S (60)[a] | 1-2 S (100) | C (100) | S (18) |
| 2 BA | 3-5 S (70) | 1-2 S (100) | C (100) | S (18) |
| 5 BA | 10-15 S (100) | >20 S (100) | C (100) | S (30) |
| 10 BA | >20 S (100) | >20 S (100) | C (100) | S (40) |
| 1 Kinetin | R (100) | R (33) | R, C (100) | A |
| 2 Kinetin | R (100) | R (30) | R, C (100) | A |
| 5 Kinetin | R (100) | R (30) | R, C (100) | A |
| 10 Kinetin | 3-5 S (100) | 4-5 S (30) | R, C (100) | A |

a: Number of shoot buds produced per explant. Figures in brackets show % explants responding.
S-Shoot bud, R-Root, C-Callus, A-Axillary sprouts.

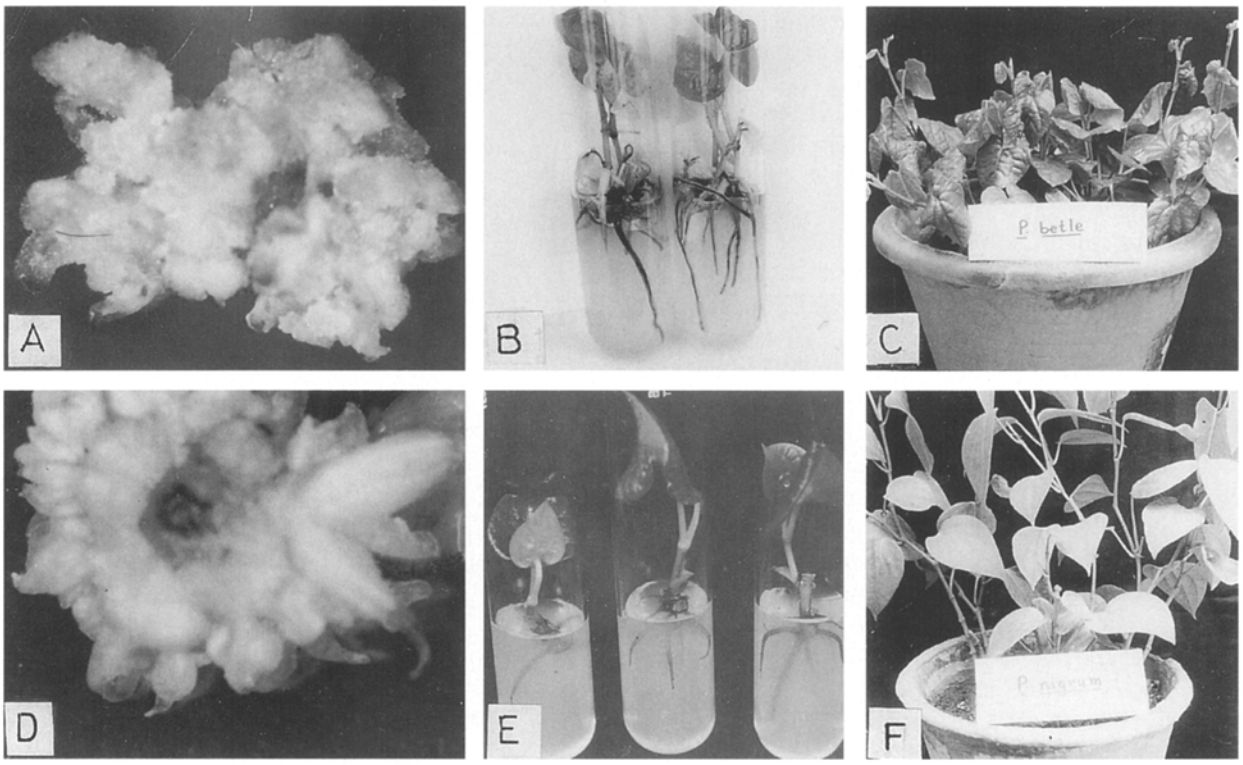


**Fig. 2.** Plant regeneration in *P. betle* (A-C) and *P. nigrum* (D-F). A, Adventitious shoot buds on an internodal explant. B, Rooted plants. C, Plants established in soil. D, Shoot buds originating around the nodal ring region. E, Rooted plants. F. Plants established in soil.

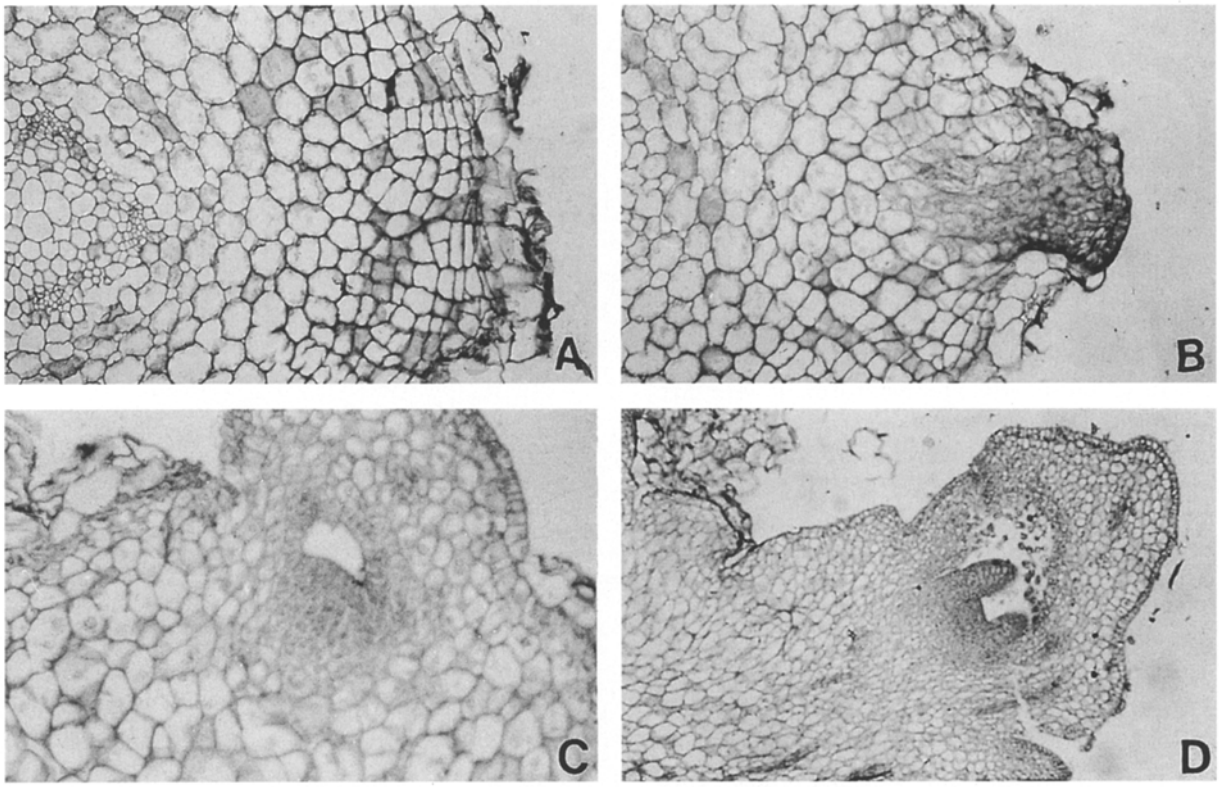


**Fig. 3.** Histology of shoot regeneration in *P. longum*. A. Transverse section of root showing meristematic activity in the cortex cells. B. Same as A, showing disruption of epidermis. C. Formation of notch like structure in meristematic cells of the cortex. D. Transverse section of an internode explant showing regeneration of shoot buds from the cortex cells.

The results clearly demonstrate that *Piper* species are amenable to *in vitro* culture. *P. longum* was the most responsive followed by *P. betle*. Further, the results highlight the importance of the choice of proper explant for regeneration. High frequency shoot bud regeneration from root explants of *P. longum* is significant because roots of relatively few plant species have been found to give rise to shoot buds in culture (Bhat *et al.* 1992; Sharma *et al.* 1993). Shoot buds on cultured root segments of *P. longum* arose directly from the cortex cells as in *Linaria vulgaris* (Bakshi and Coupland 1960). We have observed similar shoot regeneration propensity in root explants of 2 other *Piper* species, *P. hapnium* and *P. attenuatum*. Adventitious shoot bud formation from the nodal ring region of *P. nigrum* is interesting and resembles the mode of regeneration reported with the axillary buds of carnation (Miller *et al.* 1991). Further, our histological study confirmed the statement of Philip *et al.* (1992) that adventitious shoot buds arise from the stem tissue in axillary bud cultures of black pepper.

The results show that satisfactory plant regeneration can be achieved in all the 3 cultivated species of *Piper*. Because shoot buds arise from the explant without an intermediate callus phase, they may be less variable but not strictly clonal. The protocol reported here potentially will be useful in somaclonal improvement, mutation breeding and genetic transformation of *Piper* species.

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