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# 大叶种胡椒实生苗茎尖培养和合子胚培养研究

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**摘 要**：利用各种表面消毒方法对采自海南岛三个地区的胡椒大田植株的外植体进行消毒试验，由于内源性污染，除胡椒成熟种子外，其它各种大田外植体的表面消毒均未能成功。以胡椒成熟种子无菌萌发的实生苗茎尖作外植体，在 1/2MS(MS 或 $B_5$)＋1.5 mg/L BA＋0～0.2 mg/L IAA(或 NAA)上可实现丛生芽增殖。茎尖水平或竖直接种方法显著影响茎尖的增殖；水平接种茎尖的生长和增殖效果优于竖直茎尖接种方式。茎尖增殖率随 BA 浓度的增加而提高，但 BA 浓度大于 2.0 mg/L 时会使苗芽的质量降低，愈伤组织产生严重，苗芽细小，抽出不明显，颜色发黄甚至变白。附加或不附加 100 mg/L $AdSO_4$ 对丛生芽增殖没有明显影响。生根培养基以 1/2MS＋1.0 mg/L IBA＋0.5～1.0 mg/L IAA 为最优，生根率可达 100%；在细沙：土：椰糠(1：1：1)的基质中常规炼苗，成活率可达 98%以上。液体纸桥法对胡椒种胚进行培养，在不附加任何生长调节物质的培养基(MS、$B_5$ 或 SH)上只产生单苗，而在附加不同种类和不同浓度的生长调节物质的培养基上则诱导形成愈伤组织，但未能实现分化；以胡椒无菌萌发的实生苗胚轴和叶片切段作外植体进行培养，较易诱导产生愈伤组织，但难以实现分化。

**关键词**：胡椒；茎尖培养；胚培养

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# *In vitro* culture of shoot tips from seedlings and zygotic embryos of *Piper nigrum*

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**Abstract**: Experiments on surface-sterilization methods were carried out with various explants collected from field-grown black pepper (*Piper nigrum* Linn.) c. v. Daye(Lampong Type), extensively cultivated in China. Due to endogenous contaminants, contamination of all types of explants except for seeds was not yet effectively controlled. *In vitro* clonal propagation of black pepper with shoot tips excised from aseptic seedlings through multiple-shoot multiplication methods is successfully achieved. The best establishment and proliferation of shoot tips was obtained on 1/2MS(MS or Bs) basal medium supplemented with 1.5 mg/L BA and 0～0.2 mg/L IAA(or NAA). Excised microshoots were rooted *in vitro* on 1/2MS in the presence of 1.0 mg/L IBA and 0.5～1.0 mg/L IAA with the optimum rooting results. Plantlets had been successfully acclimatized and transferred to the greenhouse conditions. Complete plants were grown from mature and immature zygotic embryos of black pepper incubated on filter paper bridges in test tubes containing liquid SH(MS or $B_5$) basal medium with not any growth regulators, and calli were induced with different combinations of auxins and cytokinins. Subculture of those calli onto the multiplication medium and differentiation medium led to browning and death finally, and no plant regeneration occurred. The morphogenetic potential of other explants such as leaf pieces and hypocotyl segments from aseptic seedlings was also investigated *in vitro*. Callus induction was relatively

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easy on MS(1/2MS, $B_5$ or SH) basal medium fortified with a wide range of auxin-cytokinin combinations, but most attempts to regenerate plants from the calli were unsuccessful due to serious browning occurred during the subculture onto the multiplication medium and differentiation medium.

**Key words**: *Piper nigrum*; shoot-tip culture; embryo culture

**Abbreviations**: AC, Activated charcoal; $AdSO_4$, Adenine hemisulphate; $B_5$, Gamborg medium(Gamborg *et al.*, 1968); BA, $N^6$-Benzylaminopurine; CH, Casein hydrolysate; CM, Coconut milk; 2,4-D, 2,4-Dichlorophenoxyacetic acid; IAA, Indole acetic acid; IBA, Indole-3-butyric acid; Kt, Kinetin; MS, Murashige and Skoog(1962) basal medium; NAA, α-Naphthaleneacetic acid; PVP, Polyvinylpyrrolidone; SH, Schenk and Hildebrandt(1972) basal medium.

Black pepper(*Piper nigrum* Linn.) is an evergreen climbing shrub and one of the most important tropical spice crops. In addition to flavoring food and beverages, the fruit of black pepper is extensively used for culinary purposes such as in the treatment of asthma, pains, disease of throat, etc (Joseph *et al.*, 2001; Nybe *et al.*, 2001). Whether in volume, value or cultivation area, black pepper has been the leader, and known as "king of the spices" or "black gold" (Nambiar *et al.*, 2000). Black pepper is believed to have originated in the forests of Western Ghats of Peninsular India, and at present mainly cultivated in India, Vietnam, Indonesia, Malaysia, Thailand, Brazil, Sri Lanka, China and some other tropical countries. In China, production of black pepper is dominated by Hainan, Yunnan Province and other regions. Of those, Hainan is the largest producer of black pepper, with a production staggering around 80% of the total production in China, and its production value ranks second just after that of rubber(Cai, 1999).

*In vitro* culture technique is the basis of *in vitro* rapid clonal propagation of black pepper and the application of biotechnology such as *in vitro* selection, somaclonal variations, asymmetric somatic hybridization and gene engineering in black pepper cultivars improvement. There are few earlier reports on *in vitro* propagation of black pepper with shoot-tips of seedlings of c. v. Panniyur 1(Mathews *et al.*, 1984) and mature vines of c. v. Panniyur 1, Karimunda and Arivalli (Philip *et al.*, 1992). In addition, plantlets have been obtained from embryogenic calli derived from zygotic embryos of black pepper c. v. Panniyur 1 and Karimunda(Joseph *et al.*, 1996). Daye(Lampong Type), a black pepper variety extensively cultivated in China, is high-yielding but susceptible to foot rot. With the objective of employing *in vitro* culture techniques for rapid clonal propagation and somaclonal improvement particularly for resistance to foot rot, we first examined plant regeneration potential of various explants from the black pepper c. v. Daye.

## 1 Materials and methods

Stem segments, shoot tips, leaf, inflorescences, seeds and zygotic embryos of field-grown plants of a local black pepper variety, Daye(Lampong Type), were used as explants. From these nodal segments, leaf blades were carefully removed, leaving behind the basal stumps of the petioles covering the dormant axillary buds. Then these nodal segments were washed under running tap water for about 30～60 min. These nodal segments were surface-disinfested in a solution of 0.11% mercuric chloride for 8～15 min with frequent shaking after several seconds dip in 75% alcohol and followed by three rinses with sterilized distilled water. These nodal segments were trimmed into single node explants around 2 cm long with the petiole stumps and both ends of the nodal segments removed in a laminar air flow cabinet. The shoot tips of approximately 2 mm lengths were excised from the treated nodal segments. Immature leaves and inflorescences were surface-disinfested in a solution of 0.11% mercuric chloride for 8～10 min after several seconds dip in 75% alcohol and followed by three rinses with sterilized distilled water. Then these explants were aseptically cut

into 0.25～0.36 mm² and 5～6 mm respectively. In order to obtain seed explants, completely ripe fruits were collected and rubbed in detergent solution with the outer fleshy mesocarp removed. The seeds were washed under running tap water for 10 min and surface sterilized with 0.1% mercuric chloride for 15～20 min followed by three rinses with sterile distilled water. The zygotic embryos were dissected aseptically from surface sterilized seeds.

Due to endogenous contaminants, contamination of all types of explants except for seeds and embryos were not yet effectively controlled. Shoot tips, leaf pieces and hypocotyl segments from aseptic seedlings were actually used as explants to investigate their morphogenetic potential. These explants were aseptically cut into pieces of appropriate sizes, i. e. shoot tips (approximately 2 mm in length), leaf pieces (0.25～0.36 mm²) and hypocotyls segments (5～6 mm in length) respectively.

1/2MS, MS, SH, $B_5$ were used as the basal media and they were supplemented with various growth adjuvants such as 2,4-D (0～3 mg/L), IAA (1 mg/L), NAA (0～5 mg/L), IBA (2 mg/L), BA (0～4 mg/L), Kt (0～1 mg/L), CM (10% or 30%), CH (400 mg/L), $AdSO_4$ (100 mg/L) in different combinations and concentrations. In order to control browning and necrosis of the callus, 0.3% AC or 10% PVP was added. The pH of the media were adjusted to 5.8 prior to autoclaving and all the media were supplemented with 3% sucrose, gelled with 0.8% agar except specially noted. The culture bottles each containing 25 mL culture media were autoclaved under a pressure of 1.1 kg/cm² for twenty min. Solidified media and liquid media with filter paper bridges were used to culture the zygotic embryos. All the cultures were incubated at 26±2 ℃ under continuous illumination.

## 2 Results and discussion

### 2.1 Surface-sterilization of various explants from field-grown plants

Surface-sterilization of stem segments, shoot tips, leaf, inflorescences of field-grown plants lead to 100 percent of contamination and fungi were major contaminants responsible for the results. White hyphae still grew on the dead explants especially on cuts even surface-sterilization with exceeding 15 min led to lethality of the explants. Re-sterilization with 0.11% mercuric chloride had no positive effect on the contamination problems of the explants. Moreover, contamination was relatively higher with the seeds surface sterilized with 0.1% mercuric chloride for 15～20 min (the percentage of contamination varied between 30%～60%, sometimes to 100%). These results indicated that internal contamination was responsible for the high contamination percentage. Exogenous micro-organisms can be effectively eliminated by surface sterilants (Bonga, 1982), but endogenous contaminants are very difficult to eliminate and is a serious problem with explants of field grown trees (Arnold *et al.*, 1986; Duhem *et al.*, 1988). As for black pepper, the similar contamination problems were encountered by other researchers (Philip *et al.*, 1992). This indicated that it is important *in vitro* culturing with material free of endogenous contaminants for successful establishment of culture. The internal contamination problem was overcome by using the explants from *in vitro* grown seedlings in this study.

### 2.2 Multiplication of black pepper through *in vitro* culture of shoot tips from aseptic seedlings

Effects of different concentrations of BA and orientation of explants on shoot multiplication of *in vitro* culture of shoot tips from seedlings of black pepper were shown in Table 1. Average multiplication rate of explants was greater with shoot tips placed horizontally compared with those placed vertically. Some explants placed vertically initiated sporadic multiple shoots but most of them did produce single shoot and serious callus at all concentration levels of BA. In terms of multiplication rate, shoot growth and callus formation on the cuts of explants, horizontal orientation of shoot tips was found to be more suitable for shoot proliferation,

compared with those shoot tips placed vertically on the media in the establishment of first culture. The explants placed vertically on 1/2MS without BA eventually died. In the case of horizontal orientation, as the concentration of BA increased, the multiplication rate increased. Combination of 1.5 mg/L BA and 0～0.2 mg/L IAA (or NAA) made the newly initiating shoots more strong and healthy (Plate Ⅰ:A). While the quality of newly forming shoots was affected with the internodes of these multiple shoots highly condensed and giving a rosette-like appearance as the concentration of BA above 2 mg/L (Plate Ⅰ: B). Although maximum number of shoots per explant was observed on this medium, but the leaves of some of these multiple shoots were thick, condensed, yellow or even white with callusing tendency. Addition of adenine sulphate had no notably influence on proliferation of multiple shoots. The best establishment and proliferation of shoot tips was obtained on 1/2MS basal medium supplemented with 1.5 mg/L BA and 0～0.2 mg/L IAA (or NAA). Therefore, *In vitro* clonal propagation of black pepper with shoot tips excised from aseptic seedlings used as explants through multiple-shoot multiplication methods could be successfully achieved.

All shoots of size up to 3 cm were separated from the shoot clusters and transferred into rooting medium. Four auxin treatments with 1/2MS used as basal medium were tested. The optimum rooting results was obtained 1/2MS in the presence of 1 mg/L IBA and 0.5～1.0 mg/L IAA (Table 2, Plate Ⅰ:C). Rooted plantlets had been successfully acclimatized and transferred to the greenhouse conditions (Plate Ⅰ:D).

The orientation of the explants on the medium might have a significant effect on growth and proliferation of explants from other crops (Gunatilleke *et al.*, 1988), but the effect had not been reported in *in vitro* culture of black pepper by early researchers (Mathews *et al.*, 1984; Philip *et al.*, 1992). Horizontal orientation of the explants may have more contact with the medium and hence be helpful for absorption of nutrients through a bigger surface area. On the other hand, this orientation may have a positive effect on releasing axillary shoots from apical dominance.

Table 1 Effects of different concentrations of BA and orientation of explants on shoot multiplication of *in vitro* culture of shoot tips from seedlings of black pepper (*Piper nigrum*) (cultured on 1/2MS)

| Concentrations of BA (mg/L) | Orientation of explant inoculation | Number of shoot tips inoculated | Number of multiple shoots | Average multiplication rate |
|---|---|---|---|---|
| 0.0 | horizontal | 50 | 0 | 0 |
| | vertical | 50 | 48 | 0.96 |
| 0.5 | horizontal | 50 | 74 | 1.48 |
| | vertical | 50 | 50 | 1 |
| 1.0 | horizontal | 50 | 231 | 4.62 |
| | vertical | 50 | 53 | 1.06 |
| 1.5 | horizontal | 50 | 1104 | 22.08 |
| | vertical | 50 | 55 | 1.1 |
| 2.0 | horizontal | 50 | 1 205 | 24.1 |
| | vertical | 50 | 58 | 1.16 |
| 2.5 | horizontal | 50 | 1 213 | 24.26 |
| | vertical | 50 | 63 | 1.26 |
| 3.0 | horizontal | 50 | 1 324 | 26.48 |
| | vertical | 50 | 64 | 1.28 |

Table 2 Effect of 4 auxin treatments on *in vitro* rooting of black pepper (*Piper nigrum*) on 1/2MS

| Auxin treatments | Rooting percentage (%) | Root length (cm) | Number of roots per shoot |
|---|---|---|---|
| 1.0 mg/L NAA | 78.00 | 3.74 | 5.30 |
| 1.0 mg/L IBA | 82.10 | 4.32 | 6.10 |
| 1.0 mg/L IBA+0.5 mg/L IAA | 100 | 5.48 | 15.25 |
| 1.0 mg/L IBA+1.0 mg/L IAA | 100 | 5.20 | 14.25 |

### 2.3 *In vitro* culture of the zygotic embryos

At the end of 8 weeks of incubation of the zygotic embryos on the solidified medium with no growth regulator, the zygotic embryos just slightly swelled with different degree of browning. When transferred onto the same medium, the growth of the zygotic embryos were arrested and eventually died of browning. *In vitro* response of zygotic embryos culture on filter paper bridges in culture tube containing liquid SH supplemented with no growth regulator and different combinations of auxins and cytokinins was summarized in Table 3. With no growth regulator, the cultured embryos germinated into complete plantlets with 63.16% of germina-

tion percentage (Plate Ⅱ:E). With different combinations of auxins and cytokinins, calli were initiated and proliferated with different frequencies of callus induction(Plate Ⅱ:F). The calli could be classified into two types: one was watery, white or pale white due to browning produced with one or two auxins; the other was compact, white produced with the combinations of auxins and cytokinins. When transferred onto the same medium or 1/2 SH with no growth regulator, the callus proliferation was fast but all calli became browned and eventually died(Plate Ⅱ:G). The results showed that all calli produced in the study were with no regeneration ability. Joseph *et al*. (1996) reported that plants regenerated from embryogenic callus derived from zygotic embryos with liquid SH without growth regulators, but the zygotic embryos germinated and gave rise into single complete seedlings with same medium in our study. The reason for this might be genotype-specific *in vitro* response.

Table 3 *In vitro* response of zygotic embryos of black pepper c. v. Daye cultured on filter paper bridges in culture tubes containing liquid SH supplemented with no growth regulator and combinations of auxins and cytokinins

| Growth regulators (mg/L) | Percentage of embryos showing callus induction | Annotation |
|---|---|---|
| No growth regulator | 0 | Single plantlet produced with germination percentage of 63.16 |
| 1.0 NAA | 80.56 | — |
| 1.0 2,4-D | 82.05 | — |
| 1.0 2,4-D+1 NAA | 70.00 | — |
| 2.0 2,4-D+0.2 Kt | 94.60 | — |
| 3.0 BA+100 $AdSO_4$ | 70.83 | — |
| 2.0 BA+0.2 NAA+10%CM | 67.50 | — |
| 3.0 BA+0.2 NAA | 97.44 | — |
| 1.0 BA+0.2 NAA | 96.88 | — |

### 2.4 *In vitro* culture of leaf pieces and hypocotyl segments from aseptic seedlings

Callus induction and proliferation with the leaf pieces and hypocotyl segments from aseptic seedlings of black pepper c. v. Daye cultured on MS(1/2MS, $B_5$ or SH) basal medium added with different combinations of BA (0～15 mg/L), NAA (0～5 mg/L), Kt (0～1 mg/L), 2,4-D (0～3 mg/L), CM (10% or 30%) and/or CH (400 mg/L) were easily achieved(Plate Ⅱ:H, I). The calli were white watery, white spongy or compact slime-like. Neither direct somatic embryogenesis nor adventious shoot formation was observed, but root development was occurred on the explants directly or callus initiated from the explants when the relative concentration of auxins was higher. Following transfer to the same media or 1/2 basal medium with no any growth regulator, callus soon turned brown and necrotic and addition of 0.3 AC or 10% PVP had no effect. This phenomenon was not experienced by other researchers. With the exception of shoot tips, zygotic embryos and nodal ring tissue, plant regeneration from the explants has not been reported. The results of this study and other researchers' work showed some explants of black pepper were recalcitrant to regenerate in comparison with pipli(*P. longum*) and betel vine(*P. betel*) (Bhat *et al*., 1995; Geetha *et al*., 1990).

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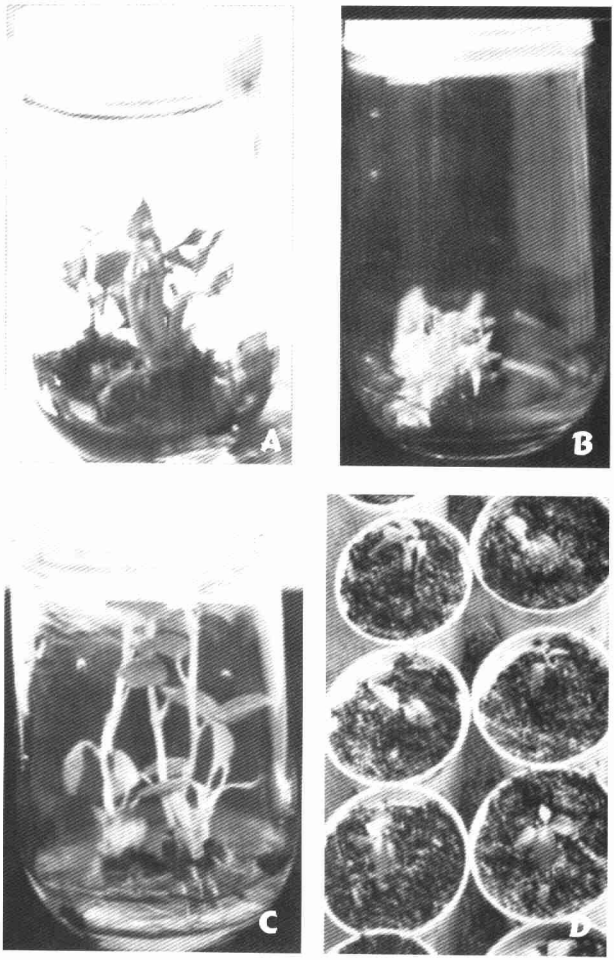
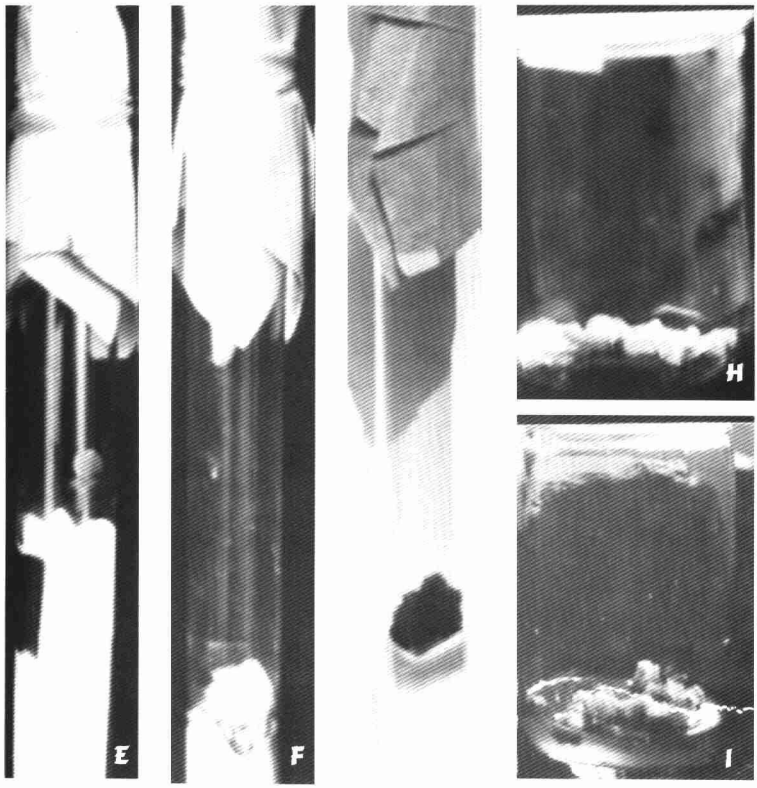



*In vitro* culture of various explants of black pepper c. v. Daye.
**A**: Multiple shoot formation in a shoot tip explant on 1/2MS supplemented with 1.5～2.0 mg/L BA; **B**: Proliferation of shoot clump in a shoot tip explant on 1/2MS supplemented with more than 2.0 mg/L BA; **C**: Rooted plantlets after 4 weeks in 1/2MS+1.0 mg/L IBA—0.5 mg/L IAA; **D**: Hardening and transferring of *in vitro* plantlets.




*In vitro* culture of various explants of black pepper c. v. Daye.
**E**: Complete plant obtained from zygotic embryo on filter paper bridge in 60 mL culture tube containing liquid basal medium without growth regulators; **F**: Callus formation on zygotic embryo; **G**: Blackening of callus initiated from zygotic embryo; **H**: Callus initiation on hypocotyl segments from aseptic seedlings; **I**: Callus initiation on leaf pieces from aseptic seedlings.