In vitro REGENERATION OF BLACK PEPPER (Piper nigrum L.)

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In vitro REGENERATION OF BLACK PEPPER (*Piper nigrum* L.)

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DECLARATION

I hereby declare that no portion of the work referred to this thesis has been submitted in support of an application for another degree or qualification to this or any other university or institution of higher learning.

( Anny Jong )

Date: 01st July 2011
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ABSTRACT

Black pepper (Piper nigrum L.) or known as King of spices, is an important cash crop of Malaysia and is widely used as food flavouring in the world. In vitro culture technique was introduced in mass production of black pepper for a newly released variety but, it is difficult to establish in vitro culture of black pepper due to the endophytic bacteria in pepper plant. Consequently, several measures are suggested in obtaining axenic culture of pepper. To begin with, the stock plants must be established from cutting planted in sterilized soil mixture and maintained under hygienic condition inside a plant-house. The explants were pre-soaking in 0.3% fungicide Benlate solution for one hour, following by surface sterilized in 10% commercial bleach (Clorox®) for 10 minutes and culture in SH medium supplemented with 0.3% PVP and antibiotic(s) tetracycline (10 mg/L) or penicillin (100 mg/L). For proliferation, shoot-tip explants with 5mm in size were cultured in SH medium supplemented with 2.5 mg/L BAP and 0.5 mg/L kinetin. Mass production of pepper plantlet via somatic embryogenesis has been very successful by cultured the seeds in SH medium supplemented with 0.5 mg/L 2,4-D. As high as 84% successful rate of acclimated plantlets were to be planted in the field to assess their field performance.

Key Words: Fungicide Benlate, commercial bleach, tetracycline, penicillin, PVP, 2,4-D, BAP, kinetin.
Regenerasi In Vitro Lada Hitam (Piper nigrum L.)

ABSTRAK

Lada hitam (Piper nigrum L.) atau dikenali sebagai Raja rempah merupakan tanaman yang penting di Malaysia dan digunakan secara meluas sebagai penyedap makanan. Teknik mikropropagasi diperlukan apabila variety baru dihasilkan. Namun, sukar untuk membangun kultur in vitro bagi lada hitam disebabkan bakteria endofit pada pokok lada. Dengan itu, beberapa langkah disarankan untuk memperoleh kultur yang aseptik. Tanaman induk harus ditanam dalam campuran tanah yang disterilkan dan dibawah keadaan bersih di dalam rumah tanaman. Hujung pucuk perlu direndam dalam larutan 0.3% fungisida Benlate selama satu jam, diikuti dengan 10% peluntur (Clorox®) selama 10 minit dan dikultur dalam medium SH ditambah dengan 0.3% PVP and antibiotik tetrakis lin (10 mg/L) atau penisilin (100 mg/L). Untuk proliferasi, hujung pucuk dengan ukuran 5mm dikultur dalam medium SH ditambah dengan 2.5 mg/L BAP dan 0.5 mg/L kinetin. Propagasi massa lada melalui embriogenesis somatik dilakukan dengan kultur benih lada dalam medium SH ditambah dengan 0.5 mg/L 2,4-D. Sebanyak 84% plantlet berjaya diaklimatisasikan dan ditanam di ladang untuk menilai prestasinya.

Kata Kunci: Fungisida Benlate, pemutih komersial, tetrakislin, penisilin, PVP, 2,4-D, BAP, kinetin.
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<td>BAP</td>
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<td>IAA</td>
<td>Indole-3- Acetic Acid</td>
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<tr>
<td>KOH</td>
<td>Potassium Hydroxide</td>
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<td>NAA</td>
<td>Naphthalene Acetic Acid</td>
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CHAPTER 1

INTRODUCTION

Pepper (*Piper nigrum* L.) which is also called black pepper is known as the ‘King of Spices’. It is the most important and widely used spice in the world for food flavouring, food processing and curing meat (Bhat *et al.*, 1995). Pepper is an important cash crop of Malaysia. Of the 284,974 tonnes of pepper produced in the world in 2009, 22,000 tonnes were from Malaysia (Anon, 2010). More than 95% of Malaysian pepper was produced from the state of Sarawak where about 13,516 hectares of land is under pepper cultivation (Anon, 2010). Domestic consumption of pepper in Malaysia is low thus most of the Malaysian pepper is exported overseas (Anon, 2009).

Pepper can be propagated both reproductively by seeds and vegetatively by cuttings taken from the orthotropic climbing stem as well as runner shoots. However, according to Philip *et al.* (1992), seedling progenies of pepper showed variation and tended to be dioecious which is not productive. Runner shoots are more difficult to strike roots and pepper plants raised from runner shoots are less productive and less precocious as compared with those raised from stem cuttings. Vegetative propagation by stem cutting thus is the preferred method. Pepper branching is dimorphic i.e. the orthotropic climbing branch which is vegetative and the plagiotropic lateral branch, which bear flower and fruit spikes. In Malaysia, a typical cutting used for propagation has five nodes taken from the orthotropic branch with two lateral branches on the two top-most nodes. One or two of such cuttings could be obtained at the first pruning, which is carried out at about six
months after planting. If the vines are grown for production of berries, the usual practice in Malaysia is to train three orthotropic shoots up the support after the first pruning. At the subsequent pruning, which is carried out at about 12 months after planting, another three cuttings could be obtained. After this, the vines are left to bear fruits and no more pruning for cuttings is practiced. However, if the plant is to be used for production of cuttings, one or two more orthotropic shoots may be trained up the support at the first pruning. Two and three rounds of pruning are carried out at the first and second year of planting respectively. There is about 15 to 25 five-node cuttings could be obtained from a plant in two years. Such rate of multiplication is considered as low. In India, runner shoots are used as planting stock but plants raised from runner shoots are less precocious and less productive. Pepper stem cuttings having any numbers of nodes can be used as the planting material. Five-node cuttings are generally considered optimal for high success in rooting, field establishment and with satisfactory growth. Cuttings with fewer nodes are slower in establishment of initial growth as compared with that of the five-node cuttings.

Demand of planting materials is high particularly when large-scale planting of a particular variety or a newly released variety is desired. Conventional method of propagation as mentioned above is inefficient to serve such purpose. A more efficient method of vegetative propagation capable of producing planting stock in large quantity is needed. Micropropagation using the in vitro culture techniques has been used in mass production of planting stock in many crops. Report of successful in vitro culture of pepper is scarce because it is hampered by microbial contamination and browning of culture. Pepper in vitro culture was successful when the explants used for culture establishment
were taken from the in vitro raised aseptic seedlings. However, the use of seedling material for mass propagation will not serve the purpose if a specific genotype is to be cloned. When field-grown materials were used, successful establishment of axenic cultures was possible if the explants were disinfected by mercuric chloride, which is toxic and not degradable in nature. The use of mercuric chloride as a disinfectant will pollute the environment. Thus, it is desirable to develop a protocol to control contamination in field-derived explant without the use of mercuric chloride.

The objective of this project is to develop a method of micropropagation for pepper. To achieve this objective it is crucial to first establish the contamination-free and viable culture. Only then induction of shoot proliferation can proceed. In the effort to achieving the objective of this project, the following strategies were formulated: (1) to control contamination from field-derived explants by subjecting the stock plants to various treatments before and after the explant materials were collected from the stock plants for in vitro culture, (2) to develop a system to establish and maintain stock plants with reduced load of microbial contaminants to provide ‘cleaner’ explant material for use in the in vitro culture, (3) to isolate and identify the microbial contaminants so the right type of biocide include antibiotic could be used to control or eliminate them and (4) to reduce browning in pepper in vitro cultures. In addition to micropropagation through induction of shoot proliferation from pre-existing buds, the pathway of plantlets regeneration through somatic embryogenesis was also explored.
2.1 Pepper vines

Pepper (*Piper nigrum* L.) is a perennial climbing plant belonging to the family *Piperaceae*. The genus *Piper* was established by Linnaeus in 1753 in his *Species Plantarum*, in which 17 species in the *Piper* family was recognized and all of which were included in the same genus (Ravindran *et al.*, 2000). Pepper is originated from the hills of South Western India, the region comprising the forests and Ghats of Kerala and the North Kanara region of Mysore up to Kanyakumari (Sim, 1985). Pepper trees are grown in the tropics of both hemispheres i.e. Western Ghats of India, North Myanmar, Indonesia, Malaysia, Brazil, Madagascar, Sri Lanka, Vietnam, Thailand and China (Farooqi *et al.*, 2005). The exact period when pepper is introduced into Malaysia is uncertain but it is believed that the Hindu colonizers of Java introduced it sometime between 100 B.C. and A.S.600. There is also no accurate record of the exact period when organized pepper growing started in Sarawak (Blacklock, 1954). Exports of pepper in 1809 from Brunei are said to have been considerable and abundant pepper gardens in the north of Sarawak were recorded in 1856 (Blacklock, 1954).

The pepper plant (Figure 1) is a perennial woody climber with luxuriant green foliage. The plant exhibits dimorphic branching; the straight, monopodial, climbing, vegetative orthotropic branches and the laterally growing, sympodial, non-climbing, reproductive plagiotropic branches. The main stem or the orthotropic shoot has indefinite
growth and produces plagiotropic fruiting branches. The orthotropic shoots climb up the living or non-living supports by means of aerial roots that arise from each node. Inflorescences arise at the node opposite a leaf on plagiotropic branch and developed into fruit spikes (Blacklock, 1954).

Pepper vines are usually propagated by cuttings. The terminal shoots of young vines that are less than two years old provide the ideal cuttings for conventional pepper propagation (Blacklock, 1954). Cultivated peppers are planted from cuttings taken from varieties that have a high ratio of hermaphrodite flowers so that full berries or the fruits produced with no blank space.
2.2 Micropropagation of *Piper nigrum* L.

Mass propagation of elite variety through *in vitro* culture technique is an extension of conventional vegetative propagation (Senawi, 1993). According to Ho (1993), in Malaysia commercial exploitation of micropropagation by the *in vitro* culture technique for mass production of elite planting materials, especially fruit and plantation crops is needed. The most significant advantage of micropropagation technique over conventional method is that large number of elite plants can be produced over a relatively short span of time and space (Ho, 1993). The techniques differ from the conventional methods by the fact it involves the use of smaller propagules, the need for aseptic and artificial environment and substantially faster plant multiplication rate (Senawi, 1993). Plant material for rapid propagation has to be taken first through meristem culture where normally up to 40% of the regenerants delivered healthy plants (Senawi, 1993). Plant regenerated from organ cultures, calli and protoplasts often show new phenotypic variability or otherwise termed as somaclonal variation (Larkin and Scowcroft, 1981 cited in Senawi, 1993). Such genetic instability may either be due to natural genetic variability that already present in the meristematic cells or induced by the culture techniques (Senawi, 1993). Besides, there are still other factors that can influence and pose problems in achieving successful rate of *in vitro* culture like endogenous bacterial contamination (Philip et al., 1992) and phenolic exudation problem (Hegde and Kulasekaran, 1996).

Regeneration protocols for many cultivated *Piper* species were reported by Bhat *et al.* (1995). Micropropagation of cultivated pepper through shoot tip cultures was reported by Mathews and Rao (1984 cited in Ravindran *et al.*, 2000) as well as by Philip *et al.*
Pepper plant regeneration through somatic embryogenesis was reported by Joseph et al. (1996). According to Ravindran et al. (2000), the first micropropagation of pepper was done by Broome and Zimmerman in 1978. In Malaysia, Chua (1981) was the first to report regeneration of plantlets from excised shoots obtained from stock plants grown in the greenhouse.

Philip et al. (1992) reported micropropagation of black pepper using shoot-tip explants taken from field-grown pepper plants. Nazeem et al. (1992) reported in vitro culture of black pepper using nodal segments of stem as the explant. In vitro culture methods for cloning of pepper using shoot tips, nodal segments and apical meristems from both juvenile and mature plants have been reported by various authors including Mathews and Rao (1984 cited in Ravindran et al., 2000), Lissamma et al. (1996) and Nirmal Babu et al. (1997). Sim et al. (1998) reported that plantlets are readily regenerated from nodal stem sections of in vitro cultured seedlings.

Somatic embryogenesis is another pathway to mass propagated pepper in vitro. According to Joseph et al. (1996), somatic embryos of black pepper can be generated from the callus derived from zygotic embryos of mature seeds. However, plantlets derived from zygotic embryos may not be true-to-type. Direct somatic embryogenesis from integument was obtained by Nair and Gupta (2003) and they suggested that such approach could be utilized for large-scale propagation and multiplication of elite genotypes.
2.3 Establishment of aseptic culture from field-derived explant

The rate of successful *in vitro* culture using shoot tip from the field-grown pepper plant as the explant material has been very low. Endogenous bacterial contamination, fungal infection and phenolic exudates released from the cut surface posed difficulty in the establishment of axenic culture (Fitchet, 1988). Bacterial contamination of explant material is the major problem in micropropagation of black pepper. According to Fitchet (1990) and Philip *et al.* (1992), *in vitro* establishment of *Piper* is greatly hampered by high incidence of bacterial and fungal contamination. Therefore, an effective surface sterilization protocol has to be established by determining the type of disinfectant, the concentration of the disinfectants and the duration of the pepper explants exposed to the disinfectant. For this purpose, mercuric chloride and commercial bleach have been found effective and used quite commonly. The commercial household bleach such as ‘Clorox*®*, is preferable as it is not toxic as the mercuric chloride, more economical and easily obtainable (Ho, 1993).

Bacterial contamination has been observed from the cut surfaces of nodal explants of ‘Kuching’ pepper variety, even after surface sterilization for 10 minutes with 0.2% mercuric chloride (Meyer *et al.*, 1992). Systemic bacteria cannot be eliminated by the commonly used methods for the initial disinfection of explants (Marino *et al.*, 1996). According to Philip *et al.* (1992), endogenous pathogens are difficult to control and they are always transferred by vegetative propagation. Although treating the explants with fungicide prior to routine sterilization, followed by frequent transfers to fresh medium after re-sterilization could reduce fungal contamination (Fitchet, 1988), bacterial contamination still remains a problem. Fungal spores apparently are released after sheath expansion and
culture the explants on filter paper bridges in liquid medium could help in reducing contamination (Philip et al., 1992).

To obtain less contaminated explants material from field-grown plants, various practices could be adopted such as growing source plants under shelter, treat the source plants with a contact and systemic fungicide to reduce both external and internal contamination, and pruning donor plants to induce new shoots, which have shorter exposure time to the open environment (Webster et al., 2003). According to Ho (1993), stock plant conditioning is necessary for the successful of establishment of axenic culture. Debergh and Maene (1981 cited in Ho, 1993) recommended the stock plant grown under carefully monitored conditions for at least three months before sampled. This stock plant conditioning stage included the precautions to reduce the level of bacterial and fungal contaminants for both surface and systemic of the explant materials (Ho, 1993). By keeping the plants in relatively low humidity (70%) and avoid an overhead watering can enhance rate of success in axenic culture initiation (Ho, 1993).

Besides, the use of antimicrobial agents to control external as well as internal contamination has been reported (Meyer et al., 1992). Antibiotic such as streptopenicillin (Lissamma et al., 1996) or tetracycline was incorporated into culture media to reduce contamination. The sensitivity of the contaminant bacteria to streptopenicillin had been proven in an earlier study by Vimi et al. (1994). Incorporation of antibiotics in the culture media was suggested by Kulkarni and Krishnamurthy (2002) to control endogenous bacterial contamination in in vitro culture of pepper. However, antibiotic only delay the
onset of bacterial growth but not to eliminate the systemic bacteria in the explant culture (Philip et al., 1992).

2.4 Endophytic bacteria in pepper plant

Biological contaminants refer to bacteria and fungi found on or within explants (Kyte and Kleyn, 1996). Endophytes were defined as microbes that colonize living internal tissues of plants without causing any immediate and overt negative effects (Stone et al., 2000). The infection of the plant with endophytic organisms may lead to improved ecological adaptability by enhancing the plant’s tolerance to environmental stresses like drought (Ravel et al., 1997) or heat (Redman et al., 2002). Plants infected by endophytes often show improved growth compared to uninfected plants (Cheplick et al., 1989), which may be in part due to the production of phytohormones like indole-3-acetic acid (IAA), cytokines (Tan and Zou, 2001) and by nitrogen fixation (Sevilla et al., 2001).

The occurrence of endophytes has been recorded from almost all vascular plants (Sturz et al., 2000), as well as from marine algae (Smith et al., 1989), mosses and ferns (Petrini et al., 1992). Fungi and bacteria seem to represent the prevalent endophytic organisms due to their presence in almost all plant species studied so far (Stone et al., 2000). The endophytic organisms may presence in the roots, stems and leaves. Roots infected with endophytic bacteria leads to an extensive and systemic spreading within the root tissue as has been shown for Penicillium sp. (Capellano et al., 1987). According to Tarkka et al. (2008), species of the genus Rhizobium can frequently be found endophytically in the roots of certain cereal crops. The endophyte Rhizobium infected the
rice root migrates from the roots to the leaves, in which enhanced growth (Chi et al., 2005). Consequently, root infection often results in enhanced plant growth (Schulz et al., 2002) especially with the presence of nitrogen fixation bacteria.

To date there is no report on the successful aseptic culture of in vitro pepper from field-derived explants using household commercial bleach as disinfectant. Field-grown pepper plant has endophytic bacteria and this has been attributed to be the main factor of the failure in establishment of in vitro pepper culture. As the bacterial contaminant appeared from cut edge at the base of the explant, Meyer et al. (1992) concluded that systemic microorganisms were present in the explant material. Moreover, endogenous bacteria had been observed in pepper protoplast culture (Sim, 2008). Recently it was reported that 66 identified strains of endophytic bacteria from six genera were isolated from pepper in India (Aravind et al., 2009). Endophytic bacteria that promote plant growth have been isolated from gramineae crop species such as rice (Zinniel et al., 2002) and sugarcane. However, there are not many reports on endophytic bacteria in black pepper.

2.5 Browning problem in pepper in vitro culture

Another critical problem in establishing in vitro culture of pepper is the release of phenolic compounds from the cut surface. Rapid browning of the medium and the explants is one of the factors causing failure of in vitro culture of Piper species. The genus Piper especially Piper nigrum contains high concentration of phenols. Phenolic compounds leached from the explants of Piper species caused browning and eventually killed the explants. This is mainly due to the presence of phenolic exudates, which was reported to