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Research Article

In vitro Derived Black Pepper Plants Used to Establish a Plantation

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ABSTRACT

Black pepper, *Piper nigrum* L, is grown in the wet tropics, almost exclusively by smallholder farmers. They generally establish new plantations using rooted stem cuttings from their own vines, which can carry diseases, and which have no identity preservation. Current black pepper production is unsustainable because of unsafe, increasingly expensive hand harvesting and prevalent use of non-compliant pesticides. Black pepper marketing companies are establishing large-scale plantations to assure sustainable compliant supplies, creating a need for true-to-type, disease-free planting material, which is presently unavailable. The objective of the study was to develop and demonstrate technology for *in vitro* multiplication of black pepper on a commercial scale and establishment of large-scale plantations of disease-free plants of desirable varieties. Production of large numbers of black pepper rooted plantlets *in vitro* was accomplished and these were airfreighted, hardened, and planted in a field plantation with a high survival rate. *In vitro* derived plants grew normally, albeit more slowly, than plants derived from rooted cuttings. Cost of *in vitro* derived plants was competitive with rooted stem cuttings.

Keywords: *Piper nigrum*; Black pepper; *In vitro* propagation; Sustainable; Disease-free; Pesticide-compliant; Commercial-scale

INTRODUCTION

Black pepper, *Piper nigrum* L., (BP), is the most important spice crop in the world with the highest total market value [1], estimated at about US$ 5 billion in 2019. Most of the crop is grown by smallholder farmers, who raise their own planting material from rooted cuttings, which are then grown attached for support to different living trees and non-living poles, made of timber, concrete, bricks or granite pillars, up to a height of 4 to 6 meters. Farmers train the vines as high as possible on these supports to maximize yield per ha. Farm-made high ladders are used on uneven terrain to harvest the peppercorns (Figure 1).

Figure 1: Harvesting black pepper. (a,b) high ladders used for hand harvesting of black pepper in Vietnam; (c) single pole harvest ladder in India.

The current system of supply of BP to the global market is unsustainable for two primary reasons. Firstly, harvesting is an unsafe and physically challenging operation for which labour is becoming less available and more expensive. Already the cost of harvesting is estimated to be 45%, 41% and 51% of total production cost in Vietnam, Malaysia, and Ethiopia respectively [2-4]. Secondly, a high proportion of BP in the world market is non-compliant with USA Environmental Protection Agency (EPA), and/or EU European Food Safety Authority (EFSA), regulations. Smallholder farmers use systemic fungicides, none of which have an EPA or EFSA tolerance for use on BP, to slow the progression of vine death caused by *Phytophthora capsici*, which attacks vines several years after planting, destroying plantations [5] (Figure 2). EFAS reported that 77% of Vietnamese BP imports were non-compliant with EU pesticide residue regulations in 2018 [6]. Since it is difficult to control the actions of smallholder BP farmers and establish traceability, two large scale marketers of BP, Olam International and McCormick Company, have commenced large-scale production of BP to establish a captive sustainable supply, with chain ownership from field to factory. An additional objective is to develop a production technology, which will allow for cost-

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effective mechanized harvest, like the development of mechanized coffee harvesting in Brazil in the early 1980's [7]. The interest by multinational food corporations in growing their own large plantations of BP has created a potential demand for large numbers of disease-free planting material of certain high-yielding varieties.

Figure 2: Destruction of 5-7-year-old vines by *Phytophthora capsici* in the major highland growing region in Vietnam

At present, rooted stem cuttings are the only planting material used to establish new plantations. Such material might be of mixed clones or varieties and could be carrying several diseases, either as localized infected tissue or in systemic infections. The two identified viruses which infect BP are cucumber mosaic cucumovirus, CMV, which causes the stunt disease, and Piper yellow mottle virus, PYMoV, a member of the genus Badnavirus, which causes pepper mottle disease. Both are readily transmitted through stem cuttings used to generate new planting material [8].

It has long been accepted that large numbers of disease-free clonal plants can be produced through tissue culture (TC), [9], and it has also been observed that some species of plants produced *in vitro* exhibit greater vegetative vigour and earlier flowering than plants derived from seed or cuttings [10]. *In vitro* propagation of BP in a laboratory was first reported in 1984 [11], from axially bud, leaf, petiole, and stem segment explants. Since then, many laboratory studies have reported successful production of rooted BP plantlets *in vitro* from different types of explant tissue. However, the only record of *in vitro* derived BP plants being used to establish a field plantation is work carried out in Pohnpei, Micronesia [12], with 200 *in vitro* derived plants, which had been hardened for 8-10 months in a nursery prior to field planting.

This study aims to develop the technology for production of *in vitro* rooted BP plantlets on a commercial scale, to demonstrate the successful planting of BP plantations using *in vitro* derived plants, and to establish the costs.

MATERIALS AND METHODS

Varieties

The main variety selected was "Kuching", a variety first selected in Sarawak. This is the dominant variety in the world market, called "Vinh Linh" in Vietnam and "Singapora" in Brazil. A comprehensive phenotypic study of 10 BP varieties grown in Malaysia showed that Kuching has by far the highest number of flower spikes per plagiotropic stem compared with the other 9 varieties and this might partly explain why it has become the preferred variety in some major BP production regions [13]. The other varieties selected were Semongok Aman, and Semongok Emas, both bred in Sarawak and released by the Malaysian Pepper Board in 1991 [14].

Explant procedures

To determine the most effective type of explant tissue, leaves, petioles, true roots, adventitious roots, stem sections, axillary buds, and apical buds were cut from carefully selected healthy vines of these three varieties growing in a plantation in Terengganu, Malaysia, and in a tropical greenhouse at Kunming Advanced Tissue Culture (KATC), facility in Yunnan, China. The tissue samples were immersed in 30-50 ppm sodium hypochlorite for 15 minutes followed by a thorough washing in 5 changes of sterile distilled water. Then, leaf discs, root tips, nodal sections, apical and axillary meristems were aseptically removed and placed individually in tubes containing slants of a medium composed of basal salts in WPM medium [15], amended with 100 mg L^{-1} inositol, 2 mg L^{-1} glycine, 1 mg L^{-1} vitamin B1, 0.5 mg L^{-1} vitamin B6, 0.5 mg L^{-1} vitamin B5, 4.5 mg L^{-1} 6 benzyl amino purine, 0.8 mg L^{-1}1-naphthaleneacetic acid, 0.5 mg/l L^{-1}, gibberellic acid, and 15 g L^{-1} sucrose. The medium was solidified by addition of 4.2 g L^{-1} agar with a gel strength 1,800 g/cm^2, and the pH adjusted to 5.8 by addition of HCl. After 2-3 weeks, the tissue which appeared to be growing and free of contaminating microorganisms was transferred into standard TC jars, 8-10 explants per jar, into the same modified WPM medium.

TC stabilization procedures

During the period February 2015 to June 2017, external contaminating microorganisms, internal endophytic bacterial contamination, (EB), and browning of the medium caused by phenolic exudates (PB), produced by wounded tissue reactions, killed most of the explants. The EB contamination problem was addressed by immersion of previously surface sterilized explant tissue in 500 ppm gentamycin solution in sterile distilled water for 2 h at 20°C 25°C [16], then rinsing in 5 changes of sterile distilled water and placing on modified WPM amended with different combinations of antibiotics, added prior to heat sterilization. The antibiotics used were streptomycin, penicillin G sodium, gentamycin, vancomycin, kanamycin, and amoxicillin [17,18]. The most consistent EB inhibition was achieved with a medium amended with 1,000 ppm streptomycin and 100 ppm penicillin G sodium, so this was used exclusively from July 2017 to present. The PB problem was addressed by adding different combinations of activated charcoal, ascorbic acid, and vitamin E together with a 50% reduction in the concentration of mineral salts.

Multiplication

Stabilized BP TC was sub-cultured into jars, 8-10 per jar, containing the same modified WPM used for the explants with the sucrose concentration increased to 30 g of sucrose L^{-1}. TC jars were placed in growth rooms and maintained at 25°C-32°C, illuminated with 1,000-1,500 lux of white, fluorescent light for 16 h d^{-1}. If any EB was seen emerging from the base of the TC stems the next sub-culture was into the same medium amended with a mixture of bacteriostatic biochemicals composed of 1.875% w/w methylchloroisothiazolinone and 0.625% w/w methylisothiazolinone isothiazolinone, 2-3 mL L^{-1} medium, plus 10 g L^{-1} of potassium sorbate [18].

Rooting procedure

Clumps of multiplying TC stems were aseptically separated and transferred to jars containing a rooting medium composed of the modified WPM multiplication medium, further modified by reduction of all the mineral salt concentrations by 50% and addition of 1 ppm of IBA.

Preparation for export to Malaysia

Rooted TC was gently removed from the jars, agar adhering to the roots was washed off with tap water at 40°C squirted from a wash bottle, and 100 each were placed in plastic trays with a moist paper towel in the base. Lids were sealed on the trays which were packed 15 each in polystyrene shipping boxes with two cool pads. The polystyrene boxes were sealed and air-freighted to Kuala Lumpur, with all required import permits and phytosanitary certificates, and thence transported by cool truck to the plantation. The total shipment period ranged from 4-6 days.

Hardening

A medium was prepared composed of 50% v/v of beach ridges interspersed with swales (BRIS), sandy soil [19], from Terengganu, Malaysia and 50% v/v coarse grade cocopeat. The medium was brought to field capacity with well water, amended with 0.5% w/w fertilizer grade gypsum and 0.1% w/w triple super phosphate, and the pH adjusted to 6.5 by addition of potassium hydroxide. The medium was placed in a layer 15 cm deep on raised benches inside an 80-mesh screened quarantine nursery with a 70% black shade net roof. The rooted TC plantlets were planted in the medium in rows 5 cm apart at a density of about 800/m^2 (Figure 3a). A clear LDPE tent, 0.05 mm thick, was erected over the plantlets (Figure 3b), which were lightly misted, and sprinkle irrigated every 2 h between 9 am-5 pm every day for the first 2 weeks when good root growth was observed (Figure 3c). Thereafter, the plants were lightly misted and sprinkled 4 times a day for the remaining time in the quarantine nursery. Environmental conditions in the tent were maintained at 28°C-33°C and 95%-99% RH. Commencing after 2 weeks, the LDPE tent was partially opened for an increasing number of hours each day until it was removed entirely about 3 weeks after planting.

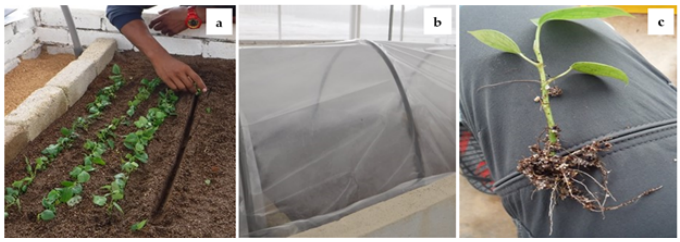

Figure 3: Transplanting of rooted TC plantlets (a) transplanting into furrows in the medium. (b) LDPE tent erected over plantlets. (c) root growth into the medium after 2 weeks.

Grow out

Hardened BP TC plants were transplanted after 30-50 d in the quarantine nursery, into black LDPE tree-bags, 8 cm diameter x 20 cm tall, filled with the same medium used for hardening and transferred to a grow-out nursery with 70% shade netting and an overhead sprinkler irrigation system. The temperature ranged from 22-33.5°C and the RH from 52-100%. The medium was wet by rainfall and sprinkle irrigated when needed. Every week a fertilizer solution composed of 200 ppm N, 50 ppm P, 70 ppm K, 60 ppm Ca, 40 ppm Mg, 30 ppm S, 1 ppm Mn, 0.5 ppm B, 0.1 ppm Cu, 0.5 ppm Zn, and 0.05 ppm Mo, dissolved in well water and adjusted to pH of 6.0, was applied until run-off was observed from the bottom of the bags. The plants were not water-stressed at any time.

Field planting

After 150-210 d in the grow-out nursery, the plants were gently transplanted into the field plantation. The soil was BRIS amended with 5% v/v compost, and the plants were set in rows 4 m apart with a 30 cm or 60 cm spacing in the rows. A drip fertigation tube, with emitters on 30 cm spacing was laid on the soil so that one dripper was close to each plant. A 70% shade net was installed at a 5 m height above the ground. It is planned to remove this net in 2-3 years, when the plants have become self-shading and able to modify their micro-environment. The plants were irrigated and fertilized on a schedule predicated by the evapo-transpiration and rainfall recorded on the site, with the same fertilizer solution used to irrigate the tree bags. Plants were sprayed with 0.1% w/w copper oxychloride at 200 L ha^{-1} every 7 d if rainfall occurred or every 14 d in the absence of rainfall.

RESULTS

Explant tissue experiments February 2015 to June 2017

Axillary and terminal meristems were the most effective explant tissues for producing TC, but the success rate was less than 1% (Table 1).

Table 1: Success rate in producing stabilized TC from different explant tissue, January 2015-June 2017

Explant tissue type	Number	Transferred into jars	Growing after 3 months	TC stabilized after 6 months	% success
Leaf discs	250	7	0	0	0.00%
Petiole	250	12	1	0	0.00%
True roots tip	100	13	0	0	0.00%
Adventitious root Tip	250	38	2	0	0.00%
Nodal section	420	58	23	1	0.24%
Axillary meristem	783	98	45	2	0.26%
Terminal meristem	702	105	50	4	0.57%
Totals	2,755	331	121	7	0.25%

Explants experiments from July 2017 to December 2017

The earlier work led to improved techniques in explant tissue selection and cutting, which raised success rate for stabilizing terminal and axillary meristem explants *in vitro* to about 8% (Table 2).

Table 2: Success rate in producing stabilized TC from terminal and apical meristem of 3 BP varieties, July 2017-December 2017.

Variety	Explant tissue	Number	TC stabilized	% success
Kuching	axillary meristem	147	9	6.12%
	terminal meristem	250	23	9.20%
Semongok Aman	axillary meristem	31	3	9.68%
	terminal meristem	28	3	10.71%
Semongok Emas	axillary meristem	17	2	11.76%
	terminal meristem	12	1	8.33%
Totals		485	41	8.45%

Control of endophytic bacteria (EB), and phenolic browning (PB), problems

EB was initially controlled by pre-soaking in 500 ppm gentamycin for 15 minutes, then sub-culturing once or twice on modified WPM amended 1,000 ppm streptomycin and 100 ppm penicillin G sodium. Subsequent emergence of EB was controlled by sub-culturing on modified WPM containing 1.875% w/w methylchloroisothiazolinone and 0.625% w/w methylisothiazolinone isothiazolinone, 2 - 3 mL L^{-1} plus 10 g L^{-1} of potassium sorbate.

The PB problem was controlled by sub-culturing 1-3 times on the modified WPM containing 1,000 ppm activated charcoal and 100 ppm ascorbic acid [20].

Multiplication phase

The multiplication rate ranged from 2.5-5 new stems from one stem in 30 days, depending on the variety and time of year (figure 4a). Over a 10-month period, Kuching TC averaged a multiplication rate of 3.5/30 d, Aman averaged 3.1/30 d and Emas averaged 2.9/30 d. By January 2020 it was projected that KATC would be able to produce 60,000 rooted BP TC from 1,000 BP TC over a 6-month period.

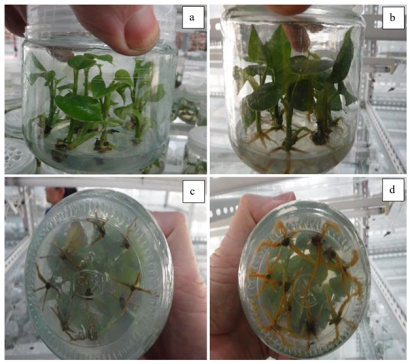

Figure 4: *In-vitro* BP multiplication and rooting. (a) Kuching stem multiplication; (b) Emas single stem rooting in 28 d.; (c) Kuching roots after 28 d.; (d) Emas roots after 28 d.

Rooting of TC stems

Strong rooting began to develop within 14 days after placing on the rooting medium and an average of 94% of TC stems developed roots in 28 days. The variety Kuching averaged 90% rooting (Figure 4b), Aman 95% and Emas 98%, all in 28 days. Emas showed stronger root development than the other varieties (Figures 4c and 4d).

Table 3: Number of BP rooted TC plants airfreighted to Malaysia from China surviving through hardening, tree-bag grow-out and planting in the field after 7 months.

Shipment	Rooted TC	Survived hardening	Planted in tree bags	Planted in field 7 months	% Survival *in vitro* to field
1st shipment	300	298	298	298	99.33%
2nd shipment	1,000	930	924	924	92.40%
3rd shipment	5,000	4,650	4,550	4,423	88.46%
Total BP survivors	6,300	5,878	5,772	5,645	89.60%

Survival from *in vitro* to growing in field plantation

The average survival from *in vitro* rooted plantlets to in vivo planted in the field was 89.6%, shown in Table 3.

Growth in the field plantation

In Malaysia, all plants grew uniformly and normally, with no sign of systemic diseases or somatic abnormalities, but they grew more slowly than plants derived from stem cuttings. The plants grew with only one orthotropic stem for 8 months, (Figure 4a), produced plagiotropic stems with flower buds in 12 months (Figures 4b and 4c).

Cost of production of *in vitro* derived BP plants

The total cost for one rooted BP TC delivered from *in vitro* production in China to the Malaysian plantation site was US$ 0.46, and the cost for one hardened grown-out plant transplanted in the plantation was US$ 0.63. A breakdown of the costs is shown in Table 4.

Table 4: Cost of growing in vitro BP rooted TC plantlets in China, based on off-take of 10,000 plantlets every 2 months, airfreighting and transport of 5,000 plantlets per shipment to a plantation in Malaysia, and cost of hardening, grow-out and transplanting into a plantation in Malaysia

RMB cost per plantlet *in vitro* multiplication and rooting	**Material**	**Labour**	**Power**	**Amortized infrastructure**	**Management & Miscellaneous**	**Total**
	0.150	1.050	0.150	0.010	0.150	1.510
RMB cost air freight, documents, local transport, packing material						0.015
RMB total cost						1.525
RMB 100% profit margin						1.525
RMB delivered price per rooted TC						3.050
US$ delivered price per rooted TC at RMB 6.6/US$ 1						0.46
US$ cost per rooted TC to harden, grow out and transplant						0.17
US$ total cost per in vitro derived BP plant established in a plantation						0.63

DISCUSSION

Research results achieved in a laboratory are a solid indication that these same results can be achieved on a commercial scale but the learning curve for scaling up with economically acceptable success rates requires time and resources. Putting explants of BP into *in vitro* TC was challenging, but after this aspect was solved, *in vitro* multiplication of large numbers was reliably achieved.

Rooted BP TC plantlets were robust and had a high survival rate through hardening and grow-out. The costs of each BP plant from third parties ready to plant into the field in Malaysia and Vietnam were US$ 1.00 and US$ 0.43 respectively [2,3]. *In vitro* derived rooted TC plants planted into a plantation cost about US$ 0.63 each, which would be competitive with rooted cuttings. However, the main advantage derived from *in vitro* propagation is the guaranteed varietal identity preservation and freedom from all diseases.

BP plants derived from *in vitro* multiplication were planted into the field in Malaysia, developed normally, but more slowly than BP plants of the same varieties derived from stem cuttings. However, Verma [21], reported that BP plants derived from *in vitro* multiplication planted in the field in Pohnpei, Micronesia produced peppercorns 12 months after planting in the field with a total yield in the second year of 988 kg ha^{-1} (Figure 5), which is more precocious and higher yielding than plants derived from stem cuttings. The climate in Pohnpei is like Malaysia but the soils are different. The soil in the Malaysia plantation was a sandy "BRIS" soil, known to have very low natural fertility and low Cation Exchange Capacity, (CEC), with no clay minerals [19,22], whereas the soils of Pohnpei were of volcanic origin and are fertile with a significant clay content and a moderate CEC. It appeared that the soil type into which BP is planted had a significant impact on the rate of plant growth and development. Further research is needed on the effect of different soil types on the growth rate of BP plants, especially the growth rate of plants derived from *in vitro* multiplication (Figure 6).

Figure 5: *In vivo* development of BP plants derived from rooted TC (a) after 8 months in the field only single orthotropic stems; (b) after 12 months in the field plagiotropic stems at top of plant with first flower buds; (c) closer view of plagiotropic stems with primary, secondary, and tertiary branches

Figure 6: Sirilanka variety of black pepper plant derived from *in vitro* multiplication, showing yield at the end of the second year, with leaves removed to better show the number of fruit spikes. Picture courtesy of Dr Virendra M. Verma, USDA Program Lead-

er and Scientist, Agriculture Production Program, Cooperative Research, Extension, and Education Services

CONCLUSION

It is technically and economically feasible to establish a BP plantation using *in vitro* propagated plantlets. It is strongly recommended that new large-scale sustainable BP plantations use exclusively *in vitro* derived plants to avoid disease problems, particularly problems from virus diseases. The plants should be raised in a nursery for 5 to 8 months and then planted on ridges or mounds in fertile, well-drained soils, in reduce the risk from *Phytophthora capsici* disease. There is evidence that yields will be more precocious and significantly higher than in plantings made from rooted cuttings. The plantations should also on smooth, gently sloping, or flat terrain which will allow for safe mechanize harvesting.

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