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In vitro Micropropagation of a Valuable Medicinal Plant, *Piper crocatum*

Zuraida A.R.[1]*, Erny Sabrina M.N.[2], Mohd Shukri M.A.[2], Razali M.[2], Norma H.[3] and Wan Zaliha W.S.[4], and Ayu Nazreena O.[1]

[1]Biotechnology Research Centre, MARDI HQ, Persiaran MARDI-UPM, 43400 Serdang Selangor, Malaysia
[2]Strategic Resources Research Centre MARDI HQ, Persiaran MARDI-UPM
[3]Horticulture Central, MARDI HQ, Persiaran MARDI-UPM, Serdang Selangor, Malaysia
[4]School of Food Science and Technology, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia 43400 Serdang Selangor, Malaysia
*Corresponding Author E-mail: azuraida@mardi.gov.my

ABSTRACT

A micropropagation protocol in valuable medicinal plant, Piper crocatum was developed which may assist in promote the industry herb in Malaysia. In the present research effect of plant growth regulators (PGRs) on shoot initiation and propagation of Piper crocatum was investigated. Shoot was initiated from internodes and apex shoots on MS medium supplemented plant growth regulator. Three type of anti-browning, proline, PVP and charcoal were tested to reduce browning problem. Internodes showed better response for shoot induction than apex shoots. MS media containing 5 mg/L BAP, 0.5 mg/L 2, 4-D supplemented with charcoal was the most effective in shoot initiation, proliferation and showed less in browning. The developed complete plantlets with root were successfully acclimatized and established in glass house and the final survival rate was 70%.

Key words: *Piper crocatum, Micropropagation, Medicinal Plant, plant growth regulator.*

INTRODUCTION

Piper crocatum is a member of the Piperaceae[22]. *Piper crocatum* L or called 'Sirih merah' as a common name in Malaysia *is* an ornamental plant with medicinal properties.

It is traditionally used as antiseptic, anti-diabetic, anticancer and to cure the infection diseases. There are also reported that plant is a traditionally used to cure various diseases such as hepatitis, stroke, kidney failure, hypertension, candidiasis etc[16,17,19,20,21] including breast cancer[11]. Red betel is one of the medicinal plants and has antibacterial activity[18]. The major categories of *P.crocatum* derived compounds that have medicinal properties are terpenoids, flavonoids, Alkaloids, saponins, and tannins[2,10]. Phytochemical screening of 'Sirih merah' leaves showed that the leaves contain chemical compounds such as polyphenols, flavonoids, tannins, alkaloids, and essential oils[24]. Polyphenols are toxic to bacteria that have the oxidized group that can inhibit the activity of enzymes in bacteria[4]. *Piper crocatum* is most commonly found in the understory of lowland tropical rainforests. It is a rare species, seldom commercially available and it is sought-out by many plant collectors. Conventional propagate of *Piper crocatum* is by stem cutting 8-10cm (3-4 inch) long. *Piper crocatum* plant is a somewhat demanding plant, steady environmental conditions being essential for its health and development. The use of plants conventionally requires large-scale plants. Nevertheless, the plant is a sturdy plant yet slows growing. Besides conventional methods of propagation, *in vitro* cultural methods contribute importantly for the propagation of many important and economic plants.

Tissue culture is one of the promising technique for effectively propagate it in large scale. Therefore, in view of the importance of this important plant, a approach for the propagation using *in vitro* methods for its conservation have been described in this paper.

MATERIALS AND METHODS

Plant materials and culture initiation

The healthy and mature of *Piper crocatum* having an age of 5-6 months-old was maintained in glass house for a few weeks prior to explants excision and establishment in vitro. After defoliation the leaves and segment of plants collected then were washed in running tap water for 1 hrs. The explants were cut into pieces of about 2-3 cm, divided into two different segments, internote and shoot apex. The segments kept in a conical flask and thoroughly rinse the tissue under running water for 1 hour. Then the plantlets were treated with the fungicide solution (Bavestin) at 5% for 1 hour, rinsed with distill water. The explants were transferred to laminar air flow chamber and were finally surface sterilized with 50% of Clorox® containing several drops of Tween-20 for 30 mins on a rotary shaker. After three rinses with sterile water, the sterilized explants were cultured onto Murashige and Skoog's (MS) (1962) medium. The MS basal medium supplemented with 3% sucrose with four different concentration of cytokinin 6-benzylaminopurine (BAP) (0, 1.0, 3.0 and 5.0 mg/L) and Kinetin (Kin) (0, 1.0, 3.0 and 5.0 mg/L). Gelrite agar was added at 3% for gelling. The pH of the medium was adjusted to 5.8 prior to autoclaving (15 min, 121°C). The cultures were incubated in the culture room under white fluorescent light with light intensity of 3000 lux at a photoperiodic/dark 12hr/12hr at 25±2°C. Cultures were checked regularly for contaminations and those presented apparent infection symptoms were immediately discarded and recorded. Data were expressed as percentage of survival of culture; shoot initiation and browning appear after 30 day of culture.

Shoot propagation and rooting

In the initial stage of culture, the explants showed higher in symptom of phenolic browning beside lower in growth and not showed any proliferation phase. The experiment were conducted to counteract browning and declining and to enhance growth and multiplication of cultures. The following factors were investigated as means for control of browning. The shoots were culture on the MS medium containing proline, polyvinylpyrrolidone (PVP) or charcoal supplemented with different concentrations of BAP (1.0, 3.0 an 5.0 mg/L) and 2,4-D (0.1, 0.2 and 0.5 mg/L) as listed in Table 2. Visual recordings were set for media browning and vigor of explants. The explants browning and browning intensity were recorded. The effect of cytokinin (BAP) and auxin (NAA) on shoot propagation was examined in a separate experiment. Single shoots were transferred onto MS or WPM medium supplemented with various concentration of BAP (0, 1.0, 3.0, 5.0 mg/L) alone or in combination with 0.2 mg/L 2, 4-D as listed in Table 3. Percentage of shoot induction, mean number of shoots and shoot length were recorded after 45 days of culture. Standard error of the mean was calculated for the degree of response which is represented in Table 1, 2 and 3 as ± value. Plantlets with root were removed from the culture bottles and the roots were washed under running tap water to remove the agar. The plantlets were then individually transplanted into polybag (containing organic soil and topsoil at the ratio of 1:1) and kept in glasshouse under 75% shading. The plantlets were watered periodically. The survival rate of the plantlets was recorded after 6-8 weeks.

RESULTS AND DISCUSSIONS

Sterilization of explants is one of the major steps for successful *in vitro* micropropagation. The step can be done by using various agents like calcium hypochlorite, sodium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide or silver nitrate[13]. The percentage of survival of cultured explants that was sterilized using 50% of Clorox® containing several drops of Tween-20 was showed in Table 1. Generally, type of explants showed the main component which internodes were more preferred compared to shoots apex. The survival rate give up to 35% for internodes compared to shoots apex only 5%. The successful of sterilization was the key for micropropagation and obtaining reduced or contamination free of the explants[5].

However, selection of sterilizing agent depends on the type of explants depending on the morphological characteristics like hardness/softness of the tissue[23]. In our study, using shoots apex was not suitable due to softness that might injure the tissue, where the explants become necrotic and die.
Similarly result was showed for shoots initiation, where internodes that culture on MS media containing 3 mg/Land 5 mg/L give the highest at 20%, respectively (Figure 1a). It was higher compared to when used shoots apex as explants. When explants of *Piper crocatum* were placed on MS basal medium with BAP or Kin, the production of phenols caused necrosis and callus turned brown and eventually died. The browning problem is a result of phenolic compounds secretion which inhibits micropropagation. Almost the explants showed 100% browning problem, except for internodes at 3 mg/L BAP (70%) and 5 mg/L BAP (80%) and for shoots apex at 3 mg/L BAP (95%). However, in this case the percentage of browning was still higher and some solutions are needed to establish the micropropagation of the plants. According Zuraida *et al.*[27], stem of Pelargonium *radula* explants performed the best on shoot initiation. They reported that with the 3mg/L BAP produced the highest number of shoot from stem explants. The explants produce globular structures then convert into axillary buds. Reported by Asaph *et al*[3], (1997), the stem explants of gypsophila originating from first internodes regenerated more shoots at ranged 74%-100%. They also found that highest shoots per explants up to 19 regenerated from the top internodes. In our result, BAP (305 mg/l) showed the best plant growth regulator compared to Kin in shoot initiation at early stage. Zuraida *et a.l*[27], MS medium supplemented with 3.0 mg/l BAP was more effective in promoting shoot in propagation of Pelargonium radula. Adel and Sawy[1] (2007), also mention about the importance of BAP for regeneration of in vitro plant. Futher more, Wong and Taha[25], study on *Allamanda cathartica* reported that MS supplemented with 5 mg/L BAP showed the best response (100%) with multiple shoots formed.

Table 1: Effect of different concentrations of BAP and Kinetin on survival rate and shoot initiation from different segment of explants after 30 days of cultured

	PGR (mg/L)		Survival rate (%)	Shoot induction (%)	Explants Browning (%)
	BAP	Kin			
Internodes	0	0	0	0	100
	0.5	0	0	0	100
	1.0	0	10±2.1	0	100
	3.0	0	30±3.2	20±2.3	70
	5.0	0	35±4.5	20±3.1	80
	0	0.5	0	0	100
	0	1.0	0	0	100
	0	3.0	5±0.5	0	100
	0	5.0	5±1.1	0	100
shoots apex	0.5	0	0	0	100
	1.0	0	0	0	100
	3.0	0	5±0.5	5±0.4	95
	5.0	0	5±0.7	0	100
	0	0.5	0	0	100
	0	1.0	0	0	100
	0	3.0	5±1.4	0	100
	0	5.0	0	0	100

Phenomena phenolic browning consequence the low multiplication rate and cultures decline in the proliferation phase. The phenolic compounds secretion which inhibits micropropagation. In this experiment was conducted to counteract browning and declining and to enhance growth multiplication of cultures. According to Nisyawati & Kusuma[15], the number of shoots produced depends on degree of explant browning. They stated that the higher degree of explant browning reduced the number of shoots produced. It was tried to be solved by applying three different anti-browning treatments in *Piper crocatum* plant species.

The three anti-browning treatments of shoot revealed the superiority of activated charcoal (AC) followed by proline treatment. PVP showed depressed to shoots and most of the shoots turned die. The result showed that explants on addition of activated charcoal in the media reduced explants browning and intensity of browning (Table 2). Moreover, more shoots propagated in this media than those on media with proline or PVP (Figure 1b). Beside, calluses also produce at the side of the explants (Figure1c) after a month of culture. In the present of BAP (3-5 mg/L) combined with 2,4-D (0.2-0.5 mg/L) in media containing charcoal was the most effective to overcome the browning beside more shoots produce.

Phenolic oxidation or brown exudate accumulation drastically decreased when activated charcoalwas used in plant tissue culture for the adsorption of inhibitory substances in the culture medium[6]. Many reported that activated charcoal may also absorb plant growth regulators, its call as a strong adsorbent[26]. It adsorbs not only toxic subtances, but also nutrients in media. Nisyawati & Kusuma[15] in their finding, of *Musa acuminata* cultured on media with addition of activated charcoal at concentration of 2g/L produced more shoots than activated charcoal in concentration lover that that. Similar observations have been reported on *Aloe vera* L, which activated charcoal stimulates growth and differentiation during culture regeneration[14].

Table 2: Effects of Anti-browning (proline, PVP and charcoal) on growth of *Piper crocatum*

	PGR (mg/L)		Explant Browning (%)	*Browning intensity	Characteristic
	BAP	2,4-D			
Proline	0	0	100	+++++	all shoots die
	1.0	0	100	+++++	all shoots die
	3.0	0	95	++++	all shoots die
	5.0	0	100	+++++	all shoots die
	1.0	0.1	90	++++	all shoots die
	3.0	0.2	50	++	callus and shoots produced
	5.0	0.5	60	++	callus and shoots produced
PVP	0	0	100	+++++	all shoots die
	1.0	0	100	+++++	all shoots die
	3.0	0	100	+++++	all shoots die
	5.0	0	100	+++++	all shoots die
	1.0	0.1	100	+++++	all shoots die
	3.0	0.2	90	++++	all shoots die
	5.0	0.5	90	++++	all shoots die
Charcoal	0	0	100	+++	all shoots die
	1.0	0	70	+++	Shoots propagated
	3.0	0	60	+++	Shoots propagated
	5.0	0	60	+++	Shoots propagated
	1.0	0.1	70	++	Shoots, callus produced
	3.0	0.2	30	+	More shoots, callus produced
	5.0	0.5	40	+	More shoots, callus produced

The intensity: +, ++ (poor) , +++ (middle), ++++, +++++ (heavy)

Different concentrations of BAP added to MS or WPM medium singly or in combination with 2,4-D affected percentage of shoot induction and number of shoots produced. The highest rate of shoot induction (75%) and number of shoots per explant (6.3) was obtained on MS media supplemented with 5.0 mg/L BAP +0.2 mg/L 2,4-D after six weeks of culture (Table 3). Small callus was observed from the leaf sheath explants on the media within 2-3 weeks time after inoculation. On the other hand, no shoots were recorded in MS medium that contained, BAP at low concentration (1 mg/l) with contained no 2,4-D. This result indicated that the addition of BAP and 2,4-D in combination further promoted the shoots induction compared to the growth regulators applied singly (Figure 1d-e). All the obtained plantlets successfully transferred into glasshouse (Figure 1f) with only 70% survival rate. Gopitha *et al.*[8] in their finding stated that application of 3 mg/L 2,4-D produce callus on sugarcane. In contrast with our result, they also observed the highest shoot regenerating capacity up to 94% was recorded in MS media containing 1.0 mg/ L BAP+0.5 mg/L NAA. Marsolais *et al.*[12] found to be the best regeneration of Pelargonium xdomesticum cv. Madame Layal when cultured in media with combination of .2 mg/l 2, 4-D with 0.5 mg/L BAP. According to Klaus-Thomas Haensch[9], the propagation of cells or differentiation of embryos inhibited for a long time by a continuous propagation on media containing both 2,4-D and BAP. Finding by Isikalan *et al.*[7] showed highest callus induction was obtained on medium containing 1.0mg/L 2, 4-D plus 1.0 mg/L BAP. Furthermore, the calli showed adventitious shoot development after it was transferred to medium containing 4.0 mg l-1 BAP only.

Table 3: Effect two types of media, MS and WPM supplemented with different concentration of BAP and 2, 4-D on percentage of shoot induction and number of shoots produced

	PGR (mg/L)		Shoot induction (%)	No.of shoot/ Explant
	BAP	2,4-D		
MS	0	0	0	0
	1.0	0	0	0
	3.0	0	35±2.1	2.1±0.3
	5.0	0	30±4.2	2.3±0.4
	1.0	0.2	30±1.1	2.0±0.3
	3.0	0.2	60±5.3	5.1±0.4
	5.0	0.2	75±6.5	6.3±1.2
WPM	0	0	0	0
	1.0	0	0	0
	3.0	0	10±2.2	1.0±0.1
	5.0	0	10±1.7	1.0±0.1
	1.0	0.2	20±3.2	1.0±0.1
	3.0	0.2	30±5.5	3.2±0.8
	5.0	0.2	30±2.8	3.1±0.5

Fig. 1: Direct plant regeneration of *in vitro* cultured *Piper crocatum*

(a) Initiation of shoots from internode (b) shoots proliferation, (c) some callus produce side the explants, (d) development of shoots, Plantlets ready to transfer into in vivo condition and (e) in vitro plant established in glasshouse condition after two months.

CONCLUSION

In this study on the *in vitro* micropropagation of the medicinal plant *Piper crocatum*, MS culture medium containing 5.0 mg/L BAP +0.5 mg/L 2,4-D that supplemented with activated charcoal gave the most suitable media for shoot initiation with less browning problem. The plantlets obtained survived and grew normally in the greenhouse. This procedure was recommended for rapid *in vitro* shoot micropropagation of *Piper crocatum*.

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