MICROPROPAGATION OF Myrmecodia Tuberosa Jack.:A MEDICINAL PLANT FROM BORNEO

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Abstract: Propagation of Myrmecodia tuberosa was developed for induction shoot from different explant sources by using various plant growth regulators (PGRs). Cotyledons, hypocotyls, tubers and roots on initiation; shoot tip and axillar bud on multiplication stages have been successfully regenerated on Murashige and Skoog (MS) medium agar. The effects of PGRs BA-6 benzylaminopurine (BAP) on initiation, BAP + GA_3-Gibberelic acid (GA3) on multiplication and NAA-α-Naphthalene acetic acid (NAA) on rooting were studied. Various medium both single and combination viz coco peat, fern, charcoal, and moss on acclimatization stage were describe. The results showed that different explant sources of M. tuberosa were potentially produced high number of shootswhich was influenced by the varying concentration of PGRs. In initiation stage, the optimum number of shoots, 15.60 per explant,was obtained from hypocotyls on MS medium agar with BAP 4 mg/L for 12 weeks while axillar bud on MS medium agar BAP 9 mg/L resulted the highest number of shoots (36.60 per explant) for 12 weeks in multiplication stage. In rooting stage, the addition of NAA 0.15 mg/L on MS medium agar was found as optimum PGRs to obtain highest number of root (18.40 root per explant). Plantlets of M. Tuberosa were also successfully acclimatized at green house in coco peat+moss and fern+moss medium in acclimatization with 100% survival. Therefore, it is suggested that the use of specific source e1xplant, PGRs either initial, multiplication, or rooting; and certain combination of medium in acclimation stage are important to be applied in order to increase the population of Myrmecodia tuberosa from North Borneo Island.

Key words: Myrmecodia tuberosa; in vitro; initiation; multiplication; acclimatization.

1 Introduction

Sarang semut (Myrmecodia tuberosa Jack), belongs to Rubiaceae and lives as an epiphyte, has an association with ants that used inner part of hypocotyl of M. tuberosa as their nest. It is an indigenous medicinal plant from Borneo island (Indonesia) and used by local people to cure various diseases. Some research regarding the effectiveness of Myrmecodia as a medicinal plant for anticancer have been studied [2; 28; 33]. M. pendens and M. tuberosa have a medical value because it contains active compounds namely flavonoids, tannins, polyphenols, tocopherols and alpha tocopherol, gaining in popularity as an antioxidant and anticancer [28; 30] The popularity Myrmecodia as a medical plant cause many people hunts directly from nature. The exploration of Myrmecodia plant without cultivation might decrease in their populations. Thus, it is an urgentneed to restoring the natural population of this valuable plant and taking conservation action through mechanisms of breeding and cultivation. Cultivation method could be used to provide a mass number of planting materials continuously and benefit for ex situ conservation of medicinal plants [3; 27]. However, there are some problems in cultivation of Myrmecodia plant such as the seeds cannot germinate well and mature seeds can be carried by ants into the ant nest cavities to be eaten [13].

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A method of plant propagation by using tissue culture techniques is an alternative way to solve these problems. Tissue culture is a method to isolate parts of the plant such as protoplasm, cells, tissues and organs, and grow them in aseptic conditions, multiplying and regenerating into whole plants. Tissue culture techniques can produce plants in large numbers and uniform [5; 10]. There are several aspects to be considered when using micropropagation tehnique such as explants material culture media (including PGRs). The level concentration and type of PGRs may vary in order to have successful tissue culture [25]. Previous research revealed that the present of PGRs, viz: cytokinins (BA-6 benzylaminopurine/BAP) were proved responsible for cell division, cell elongation and to induce shoots from the explants [24]. Meanwhile, plant propagation with different explants source has been also established in Rubiacordifolia [21], Coffea arabica [8] and Oldenlandia umbellate [24]. [32] reported that M. pendens was successfully in vitro propagated although the multiplication rate was very low. However, the information regarding the effects of Plant growth regulators (PGRs) and source of explant on the Myrmecodia multiplication is limited. Thus, the current study was design to investigate M. tuberosa propagation by using tissue culture methods with different variation of PGRs such as BAP; Gibberrelic acid (GA_3); Napthalene Acetic Acid (NAA) and different source of explant.The regeneration of M. tuberosa through several stages of initiation, multiplication, rooting and acclimatization were also described.

2. Materials and Methods

2.1 Plant material

Myrmecodia tuberosa seeds were obtained from Bulungan District, North Borneo Island, Indonesia. The seeds were sterilized with alcohol 70% for 1 min, sodium hypochlorite solution on 30%, 20%, 10% (10 min, each) and then rinsed three times (5 min, each) in sterile distilled water. For

germination, seeds were placed on MS [19], agar (0.75%) medium supplemented with 3 % sucrose.

2.2 Initiation

The cotyledons, hypocotyl, tuber and root of the 4 weeks old single seedling were used as explants for initiation. The cotyledons and tuber were scratched while the hypocotyl and roots were cut into sections of 5 mm in length. The explants were placed horizontally on MS agar (0.75%) medium supplemented with BAP at concentration 0, 2, 4, 6, 8, and 10 mg/L. After 12 weeks, the average number of adventitious shoots and shoot length were determined for each explant type and medium with PGRs. Culture conditions incubation was performed at 25 ±2 ^{0}C and 16 h photoperiod (light 36 W by fluorescent tubes).

2.3 Multiplication

The shoot tip and axillar bud (about 5 mm long) from initiation were used as explant and transferred into MS agar, containing 0, 3, 6, 9, 12 mg/L BAP and 0, 1 mg/L GA_3. The explants were placed vertically. After 12 weeks, the average number of adventitious shoots and shoot length were determined for each explant type and medium combination. Shoots were sub culture on the same treatment to media multiplication. Shoots (about 3 cm long) were used as a source of root explants. Culture conditions incubation was performed as identical as initiation stage. The individual shoot (±3 cm length) was separated and transferred to MS agar for rooting formed.

2.4 Rooting and plantlet acclimatization

Excised shoots were transferred into MS agar (0.75%) medium with 0, 0.05, 0.1 and 0.15 mg/L NAA. The cultures were incubated under the same condition as above. After 12 weeks, the percentages of shoots forming roots and the average number root per shoot were recorded. The rooted plantlets were washed in tap water to remove agar from the roots and transfered into 7 cm diameter pots, containing a sterilized medium charcoal, fern, moss, coco peat and their combination. To maintain high humidity, eachplantlet in the potwas covered with plastic glass, which was gradually opened after 3 days and completely removed after 2 weeks. The potsplantlets were kept in the greenhouse. The percentages of survival rate of the plants, the average plant length increment, and the average leave increment were recorded after 16 weeks.

2.5 Statistical analysis

Results were expressed as means ± standard error (SE), and data were subjected to analysis of varian, followed by Tukey's post hoc test to evaluate significant differences at $p< 0.05$ being the level significance. All statistical analyses were performed by using SPSS version 22 (SPSS, Inc., USA).

3 Results

3.1 Initiation

Myrmecodia tuberosa seeds that were planted in MS agar without PGRs were germinated day 3 after planting and into the whole plants in 30 days. Cotyledons, hypocotyl, tubers and roots from germination were used as a source of explants to be planted on initiation. The results showed that each explant that had been used in initiation potentially produced different number and length of shoots that caused by their genetic capability. Shoots were formed directly without callus formation that were begins with elongation of explants. After that, the small shoots were appear on the surface of explant in 2 weeks after planting. Hypocotyl was the most potent source of explants to produce of shoots (Figure 1a). The highest shoots formation (15.60±1.17 shoots and 0.1 cm of shoot length) was recorded in hypocotyls added 4 mg/L BAP (Table 1).

Figure. 1: In vitro propagation of M. tuberosa (a) Shoot production from hypocotyls on MS + 4 BAP mg/L after 12 weeks; (b). Multiple shoot formation from the nodal explants on MS + BAP 9 mg/L after 12 weeks; (c) and (d) Microshoots rooted on MS + NAA 0.15 mg/L after 12 weeks;(e) In vitro shoots established in pot after 16 weeks.

Table 1. Mean ± standard error (SE) number and length of shoot from different explants source of Myrmecodia tuberosa with various concentrations of Benzylaminopurin (BAP) after 12 weeks of culture initiation.

Type of explants	BAP (mg/L)	Shoots per explant	shoot length (cm)
Cotyledon	0 BAP	0.00±0.00^{a}	0.00±0.00^{a}
	2 BAP	4.70±0.67^{f}	0.15±0.05^{bcde}
	4 BAP	8.20±1.39^{h}	0.14±0.05^{bcde}
	6 BAP	11.00±1.24^{h}	0.16±0.05^{cde}
	8 BAP	13.10±1.19^{k}	0.15±0.05^{bcde}
	10 BAP	3.60±1.42^{ef}	0.10±0.00^{bcd}
Hypocotyl	0 BAP	0.50±0.70^{a}	0.08±0.11^{abc}
	2 BAP	6.90±1.10^{g}	0.28±0.11^{g}
	4 BAP	15.60±1.17^{l}	0.10±0.00^{bcd}
	6 BAP	3.60±0.84^{ef}	0.07±0.04^{ab}
	8 BAP	7.20±1.03^{g}	0.12±0.04^{bcde}
	10 BAP	3.50±0.52^{def}	0.10±0.00^{bcd}
Tuber	0 BAP	1.40±1.17^{abc}	0.07±0.05^{ab}
	2 BAP	6.90±1.19^{g}	0.19±0.03^{ef}
	4 BAP	4.90±0.87^{f}	0.15±0.05^{bcde}
	6 BAP	3.20±0.91^{de}	0.25±0.09^{fg}
	8 BAP	12.00±0.94^{j}	0.12±0.04^{bcde}
	10 BAP	2.40±0.51^{cde}	0.10±0.00^{bcd}
Root	0 BAP	0.00±0.00^{a}	0.00±0.00^{a}
	2 BAP	2.10±0.56^{bcd}	0.18±0.04^{def}
	4 BAP	0.00±0.00^{a}	0.00±0.00^{a}
	6 BAP	1.20±0.63^{abc}	0.09±0.03^{bc}
	8 BAP	0.00±0.00^{a}	0.00±0.00^{a}
	10 BAP	0.80±0.63^{ab}	0.08±0.06^{abc}

Means ± SE followed by the same letters in the column are not significantly different at $p < 0.05$ level according to Tukey's test

3.2 Multiplication

In multiplication, the number of shoots was affected by a combination of BAP, GA_3, and explant source (shoot tip and axillar bud). Shoots were resulted both directly and indirectly callus formation.The yellowish white callus with compact texture was formed at the base of the explant at the shoot tips and axillary buds, starting from the first week to the fourth week of observation. At the fifth week, callus started forming organogenesis in varying number. The lowest number of shoot was resulted in the media without PGRs. However the increasing concentration of BAP affected on the number of shoot.The highest number of shoots (36.60±1.52 shoot; 0.28±0.13 cm length) was obtained fromthe axillary buds added 9 mg/L BAP (Figure 1b; Table 2).

Table 2. Mean ± standard error (SE) number and length of shoot from different explants source of Myrmecodia tuberosa with various concentrations of BAP and GA_3 after 12 weeks of culture multiplication

Type of explant	PGRs (mg/L)		Shoots per explants	shoot length (cm)
Shoot tip	BAP	GA_3		
	0	0	$1.00±0.00^{a}$	$0.40±0.14^{ns}$
		1	$1.00±0.00^{a}$	$0.48±0.3^{ns}$
	3	0	$1.40±0.89^{a}$	$0.64±0.22^{ns}$
		1	$1.00±0.00^{a}$	$0.38±0.13^{ns}$
	6	0	$27.80±0.84^{f}$	$0.40±0.10^{ns}$
		1	$16.00±1.22^{c}$	$0.36±0.13^{ns}$
	9	0	$21.40±1.52^{d}$	$0.42±0.08^{ns}$
		1	$14.40±1.34^{bc}$	$0.24±0.05^{ns}$
	12	0	$14.40±1.14^{bc}$	$0.34±0.11^{ns}$
		1	$23.60±2.07^{g}$	$0.44±0.09^{ns}$
Axillar bud	0	0	$0.60±0.55^{a}$	$0.30±0.27^{ns}$
		1	$1.40±0.55^{a}$	$0.58±0.24^{ns}$
	3	0	$11.80±1.10^{b}$	$0.33±0.14^{ns}$
		1	$2.40±0.55^{a}$	$0.29±0.05^{ns}$
	6	0	$25.20±1.92^{ef}$	$0.30±0.12^{ns}$
		1	$17.20±1.64^{c}$	$0.42±0.11^{ns}$
	9	0	$36.60±1.52^{g}$	$0.28±0.13^{ns}$
		1	$22.80±2.59^{de}$	$0.40±0.07^{ns}$
	12	0	$11.60±1.14^{b}$	$0.32±0.11^{ns}$
		1	$36.20±2.39^{g}$	$0.28±0.05^{ns}$

Means ± SE followed by the same letters in the column are not significantly different at $p < 0.05$ level according to Tukey's test.
ns = non significantly different

3.3 **Rooting and plantlet acclimatization**

In the rooting stage, the average number of root was vary in each treatment (Table 3) and the addition of NAA 0,15 mg/L to MS agar resulted the highest number of root (18.40±1.29) (Figure 1c-d; Tabel 3).

After 16 weeks of acclimatization, a 100% of survival plantlet was found (Table 4), could growth well on all types of media combinations. The combination of coco peat and moss media (1:1) showed the lenghtiest plantlets (4.40±1.50 cm) (Figure 1e).

Table 3. Mean ± standard error (SE) number of root from different explants source of Myrmecodia tuberosa on MS medium supplemented with NAA after 12 weeks

PGRs (mg/L)	Root per explant
0 NAA	$7.45±0.80^{a}$
0.05 NAA	$12.90±1.49^{b}$
0.01 NAA	$16.10±1.35^{c}$
0.15 NAA	$18.40±1.29^{d}$

Means ± SE followed by the same letters in the column are not significantly different at $p < 0.05$ level according to Tukey's test

Table 4. Influence of potting mixture media on Myrmecodia tuberosa acclimatization.

Potting mixture	Survival (%)	Increase mean shoot length(cm)	Increase mean number of leaves	Increase mean number of root
Charcoal	100	3.37 ± 1.23^{ab}	3.10 ± 0.74^{bc}	6.20 ± 0.92^{a}
Fern	100	2.85 ± 0.60^{ab}	1.60 ± 0.52^{a}	7.70 ± 1.34^{ab}
Coco peat	100	3.90 ± 1.41^{ab}	2.50 ± 0.85^{abc}	8.40 ± 1.90^{ab}
Moss	100	2.73 ± 0.92^{ab}	2.10 ± 0.74^{ab}	9.90 ± 2.47^{b}
Charcoal + fern	100	3.19 ± 0.83^{ab}	2.50 ± 0.75^{abc}	6.80 ± 0.91^{a}
Charcoal + coco peat	100	3.35 ± 0.68^{ab}	1.60 ± 0.52^{a}	7.40 ± 1.17^{a}
Charcoal + moss	100	2.37 ± 0.98^{a}	1.80 ± 0.42^{ab}	7.10 ± 1.2^{a}
Fern +coco peat	100	4.20 ± 1.05^{b}	3.60 ± 1.71^{c}	7.60 ± 2.32^{ab}
Fern + moss	100	4.10 ± 1.77^{b}	5.20 ± 1.99^{d}	12.90 ± 2.42^{c}
Coco peat + Moss	100	4.40 ± 1.50^{b}	3.10 ± 0.88^{bc}	7.20 ± 0.63^{a}

*Means followed by the same letters within a column are not significantly different ($p<0.05$) using Tukey's test. Data were recorded after 16 weeks of culture.

4 Discussion

Varied response in terms on the number and length of shoot was observed as different concentration of PGRs and explant source. Current results indicated that BAP which included in the group of cytokines influenced the proliferation of shoots. This funding was in line with previous research thatBAP could increase the number of shoots on various sources of explants on medicinal plants Calendula officinalis [35] and tomato plants [18]. Furthermore, the addition of BAP to medium produces the highest shoots on the Morinda reticulata plant [20]. There are interactions between exogenous and endogenous PGRs contained in plants. The PGRs applied in production promoted growth through boosting cell division and increasing cell volume, which ascribed to comprehensive effects of many plant hormones [12]. Some plants have been successfully regenerated from various explant source such as Cunila galioides [14], Gladiolus [34], basil plants (Ocimum basilicum L.) [4], Morinda officinalis [6]. Recent finding stated that the different explants source in initiation (cotyledons, hypocotyl, tubers and roots) and multiplication (shoot tips and axillary buds) produced abundance shoots on MS agar supplemented with BAP either single or combination with GA_3. The addition of 9 mg/L of BAP resulted the highest shoot from axillar bud of M. tuberosa. This results was similar to previous research performed by [21] on Rubia cordifolia, [9] on medicinal plants Cunila galioides which also resulted the highest number of shoots on the media with BA using axillary buds as explants source. In concomitant with current finding, [17] reported that BAP was the strongest PGRs related to the multiplication of axillary buds. Similar to BAP, Gibberellin is the most commonly used hormone within the natural plant growth regulators. Gibberellin, for example GA_3, is used to enhance the length of the plant [16; 22]. Beside BAP and GA_3, NAA is an auxin group that is often used to induce the formation of roots in tissue culture [10]. The present of NAA in MS agar is effective to form roots on shoots. The present study indicated that the use of NAA 0.15 mg/L resulted the maximum number of M. tuberosa roots. Similar with this results, [11] found that the highest root formation of Rubia cordifolia was obtained by supplementing NAA. The use of auxin hormone is essential for rooting; since it promoted rooting and improved root quality in all of the used NAA concentrations, in compare to the control treated cuttings [1]. The process of root formation is affected by a number of internal and external factors. It is commonly accepted that auxinas internal factor have a specific role in the rooting initiation [22; 29]. After rooting, in vitro plantlet was transffered to acclimatization stage in order to determine the successful of in vitro plant propagation. The proper acclimatization stage can be achieved by controlling the environmental conditions. The use of humid and wet media in Myrmecodia might damage plantlet and increase their mortality. The results of the current study revealed that 100% survival of plantlets found in all various potting media. Combination of coco peat and moss medium resulted the highest number of shoot length while the optimum number of leaf and root found on combination of fern and moss medium. Previous study stated thatthe plantlets grown in potting mixture comprising the combination of fern and moss increased shoot length, leaf width and leaf number [7]. In similar way, coco peat as pure or combine withother media such as moss enhanced shoot length [15]. Coco peat being more porous substrate than other medium such as sand: soil: farmyard manure mixture. Coco peat also promoted growth of tender roots of tissue culture plants during primary hardening [26].

5 Conclusion

M. tuberosa is potential to be micropropagated by using hipocotyle source explant and BAP (4 mg/L) in initial stage, axillar bud and BAP (9 mg/L) in multiplication stage, NAA (0.15 mg/L) in rooting stage. The combination of coco peat and moss; fern and moss is recommended to be used to enhanced the number of shoot length; leaf and root number. Further research need to be done in order to determine and compare bioactive compound between tissue culture results and natural plant of Myrmecodia tuberosa.

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