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Establishment of *in vitro* mass propagation protocol in *Micropera obtusa* (Lindl.) Tang & Wang

Tapash Kumar Bhowmik[1*], Md Mahbubur Rahman[2]
[1, 2] Department of Botany, University of Chittagong, Chittagong, Bangladesh

Abstract
Endangered orchid *Micropera obtusa* (Lindl.) Tang & Wang seeds on the entire four agar solidified KC; MS; PM and MVW nutrient media with three different sources of carbohydrates *viz.* sucrose, glucose and lactose tried. Maximun seed germination (70%) was however, recorded on PM medium followed by MS (60%) and MVW (60%) media. The percentage of germination was considerably reduced on lactose supplemented KC medium (20%). Sucrose was found to be best response whereas lactose supplemented medium gave poor performance. With different concentrations and combinations of auxins, cytokinins added on agar solidified or liquid MS and PM media then germinated PLBs were cultured there. Highest shoot growth was recorded on liquid MS medium supplemented with 1.0 mg/l IAA and 1.0 mg/l BAP (2.60 ± 0.10 cm) followed by agar solidified MS + 1.0 mg/l NAA + 1.0 mg/l BAP (2.37 ± 0.15 cm). Liquid culture was superior than agar solidified condition and MS was found better than PM medium for elongation of *in vitro* grown germinated PLBs. Full strength agar solidified MS with 0.5 mg/l NAA produced highest increased length and number of roots (4.63 ± 0.28 cm/shoot bud and 2.53 ± 0.17 no/shoot bud) followed by half strength hormone free MS medium (4.03 ± 0.22 cm/shoot bud and 2.32 ± 0.12 no/shoot bud). The well-developed seedlings were transferred to outside environment and watered regularly.

Keywords: Endangered orchid; *in vitro* germination; *Micropera obtusa*; PGRs; PLBs

1. Introduction
The orchids produce numerous, microscopic, non-endospermic and poorly developed seeds which lack proper metabolic machinery to directly utilize their own lipidaceous food reserves and require a suitable fungal stimulus for germination in nature [1]. The fungus is believed to augment the carbohydrates, auxins and vitamins transport in orchids. Less than 1% seeds contact a suitable substrate to germinate in nature [1]. The technique for germinating the orchid seeds at the base of mother plant was in vogue, till in 1904 Bernard [2] suggested and successfully tested the possibility of germinating orchid seeds in the laboratory using an appropriate fungus. The possibility of by passing the fungal requirement of orchid seeds during germination by providing suitable nutrition *in vitro* [3, 4]. The resultant technique of asymbiotic seed germination has added new vistas in orchid propagation and a large number of orchid species have been successfully germinated *in vitro* [5, 9].
Micropera obtusa (Lindl.) Tang & Wang distributed in India, Bhutan, Nepal, Myanmar and Thailand. It has horticultural potential for its showy tiny flowers [10] and flowering time August [11, 12]. *M. obtusa* is rare orchid of Bangladesh for indiscriminate collections by orchid lovers, habitat destruction, over exploitation by orchid lovers [10]. For that, the species has detrimentally affected the size and frequency of its natural populations. The present paper reports the germination of its seeds and development of seedlings with a view to developing a protocol for its mass multiplication.

2. Materials and Methods
Collected mature green capsules of *Micropera obtusa* were washed in the running tap water for 5- 10 minutes to remove dust in the surface area. Then few drop of teepol solution was added for few minutes and washed under running tap water for five minutes. The capsules were surface sterilized by 0.1% $HgCl_2$ for 10 minutes followed by 70% ethanol for 30 second. Washing thoroughly with sterile distilled water to free the capsule of any traces of the sterilizing agents.
The seeds were inoculated on agar gelled four basal media namely, KC [13]; MS [14]; PM [15] and MVW [16] with three different sources of carbohydrates *viz.* sucrose, glucose and lactose for germination. The pH of all media was adjusted to 5.8 prior 0.1N NaOH or HCl before mixing agar to autoclaving at 121^0C for 20 minutes at 15 lbs pressure. Seed germination was carried out in the culture room at 25±2 ºC and under white fluorescent tubes at an intensity of 4000-5000 lux with a 14/10-h (day/night) photoperiod [5]. Hormones *viz.* BAP, Kn, Pic, NAA, IAA and IBA were freshly prepared. All The experimental manipulation was carried out under aseptic conditions and experiment is repeated thrice.
For the inoculation of seeds, mature green capsule was put on sterile tile and cut longitudinally using a sharp sterile blade under laminar air flow cabinet. The very minute seeds were scooped out with the help of sterile forceps and spread over the surface of the germination media. Full strength MS and PM based eighteen different solid & liquid elongation media were prepared using with different concentrations and combinations of PGRs. 0.8% (w/v) agar was also used in solid media but in liquid media no agar was used. For induction of strong and stout root system, *M. obtusa* seedlings cultured on agar solidified half strength MS0 with 1.5% (w/v) sucrose and full strength nine different types of MS medium fortified with 3% (w/v) sucrose with three auxins *viz.* IAA, IBA and NAA. The well developed seedlings were taken out of the culture vessels and successfully transferred to outside the culture room following successive phases of acclimatization and transplanted seedlings were watered regularly for about 2-3

months.

3. Results and Discussions

The seeds of *Micropera obtusa* germinated on all the four-nutrient media like KC, MS, PM and MVW supplemented with various carbohydrate source *viz*. glucose, lactose and sucrose (Table-1). Maximum seed germination was recorded on sucrose containing PM medium (70%; Fig.1a) and it was followed by that in MS (60%; Fig.1b) and MVW (60%) media. The germination was significantly impaired on lactose containing KC (20%) and MVW (20%) medium. The time depends on different nutrient composition on the media. Similar result was also found in *Ponthieva* and *Cattleya* [17]; *Cymbidium bicolor* [18]; *Cymbidium aloifolium* [19]*; Dendrobium aphyllum* [20]; *Geodorum densiflorum* [21]; *Arundina graminifolia* [22] and *Calanthe densiflora* [23] orchid species. PM medium also proved more effective for inducing early and better germination of seeds. PM media is enriched with vitamins and organic additives. Addition of vitamins and additives into the medium was reported to be enhanced for seed germination and seedling growth of many orchids. Peptones has significant role as excellent natural sources of amino acids, peptides and proteins in growth media. Peptone in media enhances the germination rate and also favours the healthy protocorm development. Seed germination as well as protocorm growth and development were better on MS medium. MS medium was found best for germination in *Dendrobium densiflorum* [1]; *Dactylorhiza hatagirea* [9] and *Vanda* [24]. Higher concentrations of nitrogen present in MS medium was required for the optimal germination of seeds [1].

Table 1: *In vitro* germination of seeds of *Micropera obtusa* (Lindl.) Tang & Wang

Nutrient medium	Carbohydrate source with concentration (w/v)	Number of culture vessels used	Number of culture vessels in which seeds germinated		Time (d) required for germination	Remarks
			No.	%		
KC	2% glucose	10	03	30	38 - 42	Yellowish green PLBs
	2% lactose	10	02	20	35 - 40	Whitish green PLBs
	2% sucrose	10	05	50	33 - 35	Green PLBs
MS	3% glucose	10	05	50	35 - 40	Yellowish green PLBs
	3% lactose	10	03	30	30 - 35	Whitish green PLBs
	3% sucrose	10	06	60	35 - 38	Green PLBs
PM	2% glucose	10	05	50	40 - 42	Whitish green PLBs
	2% lactose	10	04	40	40 - 45	Whitish green PLBs
	2% sucrose	10	07	70	32 - 36	Green PLBs
MVW	2% glucose	10	05	50	34 - 36	Yellowish green PLBs
	2% lactose	10	02	20	38 - 42	Whitish green PLBs
	2% sucrose	10	06	60	32 - 35	Green PLBs

Sugar is an important component of any kind of nutrient medium used in tissue culture studies. *In vitro* plant cells, tissues and organ cultures are not fully autotrophic establishing a need for carbohydrates in culture media to maintain the osmotic potential, as well as to serve as energy and carbon sources for developmental processes including shoot proliferation, root induction as well as emission, embryogenesis and organogenesis, which are highly energy demanding developmental processes in plant biology. A variety of carbon sources (Monosaccharide and Disaccharide) are used in culture media depending upon genotypes and specific stages of growth. However, sucrose is most widely used as a major transport-sugar in the phloem sap of many plants. In germination and micropropagation systems, morphogenetic potential of plant tissues can greatly be manipulated by varying type and concentration of carbon sources [25]. Our results indicate that selection of medium is an important aspect of success in asymbiotic germination of this orchid species.

The *in vitro* developed protocorms on the basal media produced tiny seedlings on following subculture in the same media and their growth rate was very poor. But when the protocorms were grown on eighteen types of with various combinations, concentrations of PGRs (BAP, IAA, NAA, and Picloram) solid & liquid MS and PM media gave differential responses. No agar was added in liquid media. Different hormone combinations and culture condition were found to be better for elongation of seed originated small plantlets (Table-2). The rate of elongation of seedlings after 30d of culture was dependent on the PGRs combinations,

Table 2: Elongation of *in vitro* germinated seedlings of *M. obtusa* on 0.8% (w/v) agar solidified and liquid media with different kinds of PGRs.

Culture medium with different combinations and concentrations of PGRs	Average initial length (cm) of *in vitro* germinated seedlings	Average length (cm) of germinated seedlings after 30d of culture on elongation medium	Increase in length (cm) of germinated seedlings within 30d of culture on elongation medium	Average initial length (cm) of *in vitro* germinated seedlings	Average length (cm) of germinated seedlings after 30d of culture on elongation medium	Increase in length (cm) of germinated seedlings within 30d of culture on elongation medium
	Solid media			Liquid media		
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.65±0.15	3.74±0.13	2.09±0.11	1.40±0.16	3.85±0.17	2.45±0.19
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.62±0.12	3.53±0.15	1.91±0.16	1.45±0.13	3.57±0.10	2.12±0.12

MS + 3% (w/v) sucrose +1.0 mg/l IAA + 1.0 mg/l BAP	1.68±0.16	3.73±0.14	2.05±0.19	1.42±0.14	4.02±0.12	**2.60±0.10**
MS + 3% (w/v) sucrose +1.0 mg/l NAA + 0.5 mg/l BAP	1.42±0.10	3.53±0.14	2.11±0.12	1.52±0.09	3.89±0.12	2.37±0.11
MS+3% (w/v) sucrose + 0.5 mg/l NAA+1.0 mg/l BAP	1.52±0.09	3.60±0.12	2.08±0.10	1.50±0.15	3.55±0.10	2.05±0.13
MS + 3%(w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	1.55±0.13	3.92±0.11	2.37±0.15	1.48±0.12	4.00±0.11	2.52±0.15
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.65±0.14	3.97±0.12	2.32±0.15	1.50±0.10	3.98±0.13	2.48±0.14
MS+3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.50±0.17	3.54±0.15	2.04±0.13	1.55±0.11	3.72±0.14	2.17±0.12
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.55±0.15	3.80±0.13	2.25±0.18	1.55±0.18	3.97±0.13	2.42±0.16
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.48±0.19	3.84±0.14	2.36±0.15	1.61±0.08	4.01±0.12	2.40±0.10
PM + 2% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.65±0.11	3.66±0.10	2.01±0.14	1.55±0.16	3.79±0.14	2.24±0.12
PM + 2% (w/v) sucrose +1.0 mg/l IAA + 1.0 mg/l BAP	1.75±0.16	3.99±0.15	2.24±0.12	1.42±0.15	3.78±0.11	2.36±0.12
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.60±0.13	3.89±0.11	2.29±0.16	1.45±0.19	3.93±0.15	2.48±0.13
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	1.70±0.14	3.83±0.09	2.13±0.12	1.40±0.12	3.49±0.14	2.09±0.09
PM + 2% (w/v) sucrose +1.0 mg/l NAA + 1.0 mg/l BAP	1.62±0.08	3.89±0.11	2.27±0.13	1.50±0.15	3.92±0.13	2.42±0.11
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.55±0.07	3.89±0.09	2.34±0.11	1.52±0.16	3.99±0.18	2.47±0.13
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.80±0.12	3.88±0.14	2.08±0.16	1.45±0.13	3.58±0.14	2.13±0.17
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.75±0.10	3.87±0.12	2.12±0.08	1.52±0.11	3.90±0.17	2.38±0.15

*All the values are mean ± SE, shoot length of each treatment contains 10 replicates.

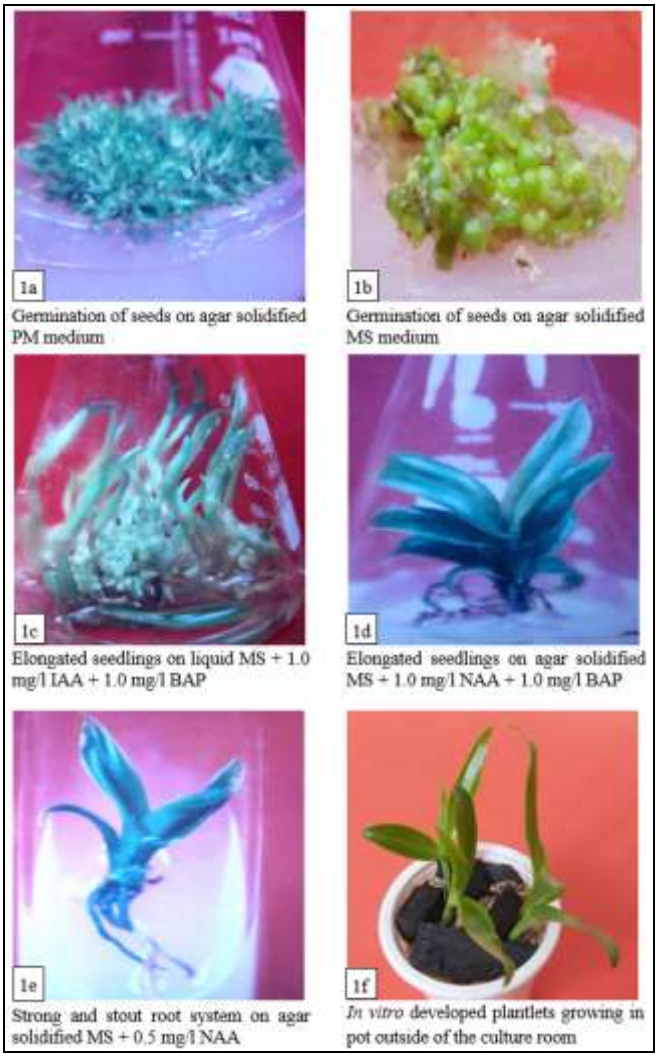

Fig 1 (1a-1f): *In vitro* germination, elongation, rooting and hardening of *Micropera obtusa* (Lindl.) Tang & Wang

concentrations and culture conditions. Maximum elongation took place on liquid MS medium fortified with 3% (w/v) sucrose + 1.0 mg/l IAA and 1.0 mg/l BAP (2.60 ± 0.10 cm; Fig. 1c) followed by agar solidified MS medium with 3% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP (2.37 ± 0.15 cm; Fig. 1d). It is reputable that elongation of seed originated seedlings was better in liquid media than agar solidified condition. Further MS was superior than PM for elongation of shoot bud [18, 21, 23, 26].

The elongated seedlings at a height of 3-4 cm length were individually grown on half strength MS0 and nine different types of PGR (IAA, IBA, NAA) supplemented MS media were used for induction of strong and stout root system (Table-3). MS medium fortified with 3% (w/v) sucrose + 0.5 mg/l NAA produced highest increased length and number of roots (4.63 ± 0.28 cm/shoot bud; 2.53 ± 0.17 no/shoot bud and Fig. 1e) followed by half strength hormone free MS medium + 1.5% (w/v) sucrose (4.03 ± 0.22 cm/shoot bud and 2.32 ± 0.12 no/shoot bud) was proved best for induction of strong and stout root system within 30d of culture. Similar result was found in *Esmeralda clarkei* and *Vanda tessellata* [27-28]. IBA was effective for rooting in *Ilex khasiana* and *Cymbidium finlaysonianum* respectively [29-30]. The opposite result was also noted that IAA was most appropriate in inducing roots in *Dendrobium* hybrid, *Dendrobium thyrsiflorum* respectively [31-32]. Combine effect of IAA, IBA or NAA induced excellent rooting response in *Rhyncostylis retusa* and *Aerides ringens* [33-34] orchid species.

Table 3: Increased length (Mean ± SE) and number of roots in seed derived seedlings of *M. obtusa* in half strength MS0 and auxin supplemented MS rooting media.

Culture medium			Average increased length and number of roots per seedling	
			Mean length (cm) ± SE	Mean no. of roots/ shoot bud ± SE
½ MS0			4.03 ± 0.22	2.32 ± 0.12
Auxin (mg/l)	IAA	0.5	3.59 ± 0.21	1.87 ± 0.14
		1.0	3.72 ± 0.23	2.09 ± 0.13
		1.5	3.94 ± 0.26	2.21 ± 0.17
	IBA	0.5	2.17 ± 0.15	1.04 ± 0.07
		1.0	3.23 ± 0.24	1.76 ± 0.12
		1.5	3.89 ± 0.25	2.11 ± 0.14
	NAA	0.5	4.63 ± 0.28	2.53 ± 0.17
		1.0	3.01 ± 0.17	2.24 ± 0.18
		1.5	2.74 ± 0.19	1.71 ± 0.12

*Root length and number of roots of each treatment contains 10 replicates.

Established rooted plantlets were transferred from culture room to the green house during successive phase of adjustment (Fig. 1f). For this purpose, the culture vessels were kept open for one day in the culture room and then kept outside of the culture room for 6h in the next day. On

the third day those were kept outside of the culture room for 12h. Finally the seedlings were taken out of the culture vessels and rinsed with running tap water for removal of agar attached to the roots. Then the seedlings were transferred to plastic pots containing a potting mixture of sterilized small brick, coal pieces, saw dust and peat moss at a ratio of 1 : 1 : 1 : 0.5 and kept in the green house (at 25-30 ^{0}C and RH 60-70%). Transplanted seedlings were watered regularly for about 2-3 months and established in the Orchidarium.

4. Conclusions

PM was found superior than KC, MS & MVW media for promoting germination of *M. obtusa* seeds and sucrose supplemented media was best than lactose and glucose as a source of carbohydrates. Enhancing elongation of *M. obtusa* seedlings, liquid culture was better than agar solidified condition. Increased in root length and number of roots is higher in NAA supplemented full strength MS followed by hormone free half strength MS medium. *Ex situ* conservation of this species is highly recommended to reduce its vulnerable condition.

5. References

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