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In Vitro Secondary Embryogenesis Derived from Meta-Topoline Treatment on Mass Propagation of *Phalaenopsis* 'AMP 17'

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Abstract

Phalaenopsis 'AMP 17' is an important orchid commodity in Indonesia with high market demand; however, scaling up the orchid commercially is constrained by the availability and sustainability of qualified seedlings. To overcome the problem, a reliable *in vitro* propagation protocol, especially via secondary embryogenesis, was undertaken. In the present study, *in vitro* secondary embryogenesis derived from meta-topoline (mT) treatment on mass propagation of *Phalaenopsis* 'AMP 17' was successfully established. Embryos, as explant sources, were prepared by culturing meristem tips of flower stalk shoots on Murashige and Skoog (MS) medium containing 1.5 mg/L thidiazuron (TDZ) and 0.25 mg/L N^6-benzylaminopurine (BAP) for ± 3 months. High secondary somatic embryo (SSE) formation up to 64.90% with 12.30 SSEs regenerated per embryo was determined on half-strength MS augmented with 0.5 mg/L BAP and 2.5 mg/L mT. The combination also stimulated the result of high multiplication rate of SSE formation, up to 10.1 fold on the third subculture, maintained low conversion rate of germinated-embryos down to 55% and improved qualified-growth of the germinated embryos. The mT treatment produced 86% survival plantlets with high qualified-performance. The system could be applied as an alternative method to step forward towards an improved propagation protocol, commercially efficient due to high productivity. Detail findings in each step were discussed.

Keywords: hydroxylation benzyladenine, moth orchid, multiplication rate, repetitive embryogenesis, secondary somatic embryo, SSE

Introduction

Phalaenopsis is one of important orchid commodities in the world, with economically impact in Indonesia. The orchids have various flower types, sizes, colours and in some cases fragrance (Tang and Chen, 2007). *Phalaenopsis* are generally sold as cut flowers and pot plants with different prices depending on cultivar and decorative quality, respectively. The orchid varieties cultivated and commercially produced in Indonesia are mostly imported, 89% are consumed by local market, while 11% are exported to Japan, USA, Netherlands, Singapore, Australia and Canada (Wisdya, 2009).

Development of the *Phalaenopsis* in a commercial scale is significantly constrained by the availability and sustainability of high qualified-seedlings. Thus, a reliable mass propagation protocol for the moth orchid shall be addressed.

However, *in vitro* mass propagation protocols for *Phalaenopsis* were successfully established using varied-explants, basal media, concentrations and combinations of plant growth regulators (PGRs), with good results for different varieties (Murdad *et al.,* 2006; Sinha and Jahan, 2011; David and Bala, 2012; Samarfard *et al*., 2013; Feng and Chen, 2014; Rittirat *et al*., 2014). Each plant genotype and explant has specific behaviour within *in vitro* culture in response to medium composition and other factors. Therefore, each genotype needs specific *in vitro* propagation route system; one of the route systems is somatic embryogenesis, as established by Kuo *et al*. (2005), Chen and Chang (2006), Gow *et al*. (2010) and Feng and Chen (2014), while specific studies concentrating on secondary somatic embryogenesis are scarce. The formation of secondary somatic embryo (SSE) on *Phalaenopsis* was regenerated on basal part of somatic embryos (SEs) (Kuo *et al*., 2005), nodular masses of primary embryos (Chen and Chang, 2006) and embryo section (Park *et al*., 2010), especially under thidiazuron (TDZ) treatment with a pivotal role in the formation of SSEs. However, long term application of TDZ induced hyperhidricity, morphological alteration and somaclonal variation as reported for *in vitro* culture of banana (Bidabadi *et al*., 2010) and *Phalaenopsis* (Khoddamzadeh *et al*., 2010). However, meta-topolin (mT) and its advantages in stimulating SSE are still to be studied.

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Meta-topolin (mT) [6-(3-hydroxybenzylamino) purine] is a hydroxylation benzyladenine with high activity for morphogenesis isolated from the leaves of *Populus* × *canadensis* (Strnad *et al*., 1997). Application of mT can improve some physiological disorders (Aremu *et al*., 2012) and decrease the rate of shoot tip necrosis (Amoo *et al*., 2011). Successful application of mT in different purposes of *in vitro* culture was also reported previously in pineapple (Teklehaymanot *et al*., 2010), *Citrus reticulate* × *Poncirus trifolia* (Niedz and Evens, 2011), *Prunus domestica* L. and *Peunus institia* × domestica (Gentile *et al*., 2014). However, application of mT in order to induce secondary embryogenesis in *Phalaenopsis* 'AMP 17' has not been reported so far.

The objective of the present study was to establish a reliable *in vitro* mass propagation protocol of *Phalaenopsis* 'AMP 17' via secondary somatic embryogenesis based on mT treatment on selected media. The study was undertaken starting with the initiation of SSE formation, followed by multiplication and conversion till acclimatization. Interesting findings in several studies using the mT treatment are discussed in this paper.

Materials and Methods

Planting material and sterilization

Phalaenopsis 'AMP 17' was used as donor plant in the study. The harvested floral stalks were pre-treated by cleaning and weeping the floral stalk with 96% alcohol wetted cotton, placing them under tap water for 60 min, immersing in 1% detergent for 30 min and rinsing with distilled water 4-5 times (3 min each). The stalks were then cut individually (at each node) and prepared as explant sources. The explants were then separately disinfected by soaking them in 0.05% and 0.01% mercury chloride ($HgCl_2$) for 5 min and finally rinsed with sterile distilled water for 5 times (aprox. 5 min each). The sterile explants were subsequently cultured on the explanting medium.

Explant preparation

Preparation of embryos as explant sources was carried out by culturing the prepared nodes on MS medium supplemented with 0.5 mg/L TDZ, 0.25 mg/L BAP, 20 g/l sucrose and 7 g/l Swallow agar and incubated under light incubation for 16 h photoperiod under cool fluorescent lamp with 13 μmol/m^2/s light intensity at 24 ± 2 °C for ± 8 weeks. The meristem tips of the shoots derived from the node explants were then carefully isolated by removing scale-like leaves that covered them, one by one, using sharp tissue culture blade. After removing all scale-like leaves, the small shoot tip (0.3 mm in length, width and height) was sliced and cultured on MS medium fortified with 1.5 mg/L TDZ, 0.25 mg/L BAP, 20 g/l sucrose and 2.0 g/L gelrite for ± 1.5 months, in dark conditions; here after, the explants were transfer to light incubation till vigor embryos were produced (± 1.5 months). The embryos derived within this stage were then used as explant sources for the current study.

The effect of selected media and mT on SSE formation

The two selected media tested in the hereby experiment were SM1 (half-strength MS full vitamins) and SM2 (half-strength MS supplemented with 0.5 mg/L BAP), while the various concentrations of mT were 0.0, 0.5, 1.0, 2.5, 5.0, 7.5 and 10.0 mg/L. All media used in the study contained 2% sucrose and 2.0 g/L gelrite. The experiment was arranged in a randomized complete block design (RCBD) with 4 replications. Each treatment consisted of 2 petri dishes (5 cm in diameter). Each petri dish contained 5 clustered embryos and each cluster had ± 5 embryos. Cultures were incubated in dark condition for ± 8 weeks.

Periodical observations were carried out to note explants alteration during the incubation interval. Parameters observed in the experiment were percentage of SSE formation and the number of SSE per embryo, and were counted ± 8 weeks after culture establishment.

Determining the effect of selected media and mT concentrations on multiplication and conversion of SSEs

Two selected media and different concentrations of mT as described above were applied in the experiment. The embryos were sub-cultured for 4 times and observations were registered monthly until embryo germination (S1, S2, S3, S4). Parameters recorded were (1) embryos fresh weight (g), (2) number of SSE and (3) frequency of SSEs conversion (%).

Determining the effect of selected media and mT concentrations on plantlet growth

Small plantlets (± 0.4 cm plant height; ± 2.0 leaves; ± 0.41 cm^2 leaf area; ± 0.6 roots; ± 0.5 root length) were used in the study. The two selected media were (1) SG1: half strength MS medium with full vitamins and without activated charcoal (AC) and (2) SG2: half strength MS medium augmented with 1.5 g/L AC. Similar concentrations of mT (as for SSE formation) were applied within the experiment. The factorial experiment was arranged in RCBD with 4 replications. Each treatment consisted of 2 jam bottles (7 cm in diameter, 12 cm in height and 30 ml of medium). Each bottle contained 5 plantlets. Parameters observed were (1) number of shoots, (2) plant height (cm; measured from the basal part of plantlet to shoot apical meristem), (3) number of leaves per plantlet, and (4) number of roots per plantlet. All data were collected after ± 8 weeks of culture.

Determining the effect of mT concentrations on plantlets acclimatization

Rooted plantlets (± 0.4 cm height; ± 2 leaves in average, ± 0.85 cm^3 leaf area, ± 1.5 root, ± 1.0 cm root length and ± 40 SPAD unit of leaves chlorophyll content) derived from the different mT treatments established within the experiment were used in the acclimatization stage. The acclimatization of plantlets was started by taking plantlets from the jam bottles, cleaning the root plantlet from remains of agar, soaking the roots in 1% pesticide solution (50% benomil and 20% kanamycin sulphate) for 3 min, then planting them separately based on mT treatments in plastic trays (2 cm in diameter). The trays were then placed in glass house under low light (87-118 μmol/m^2/s) and humid area (65-80%) without watering for 7 days. Each treatment contained 5 replications, and each tray contained 5 plantlets, thus obtaining a total of 125 acclimatized plantlets. After 8 weeks of acclimatization, (1) percentage of survivability (%), (2)

Table 1. The effect of selected medium on SSE formation

Selected medium	Percentage of SSE formation (%)	Number of SSE per embryo
SM1[1]	48.98 b	6.60 b
SM[2]	63.75 a	11.98 a

SM1: Half-strength MS full vitamin; SM2: Half-strength MS supplemented with 0.5 mg/L BAP. Means followed by the same letter in the same column are not significantly different (Duncan multiple range test, P=0.05)

Table 2. The effect of different concentrations mT on SSE formation

mT concentration (mg/L)	Percentage of SSE formation (%)	Number of SSE per embrio
0.0	44.36 bc	5.99 ab
0.5	39.92 c	5.62 b
1.0	57.57 ab	8.62 ab
2.5	64.86 a	12.26 a
5.0	63.40 a	11.66 ab
7.5	63.12 a	11.43 ab
10.0	61.30 a	9.43 ab

Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test, P=0.05)

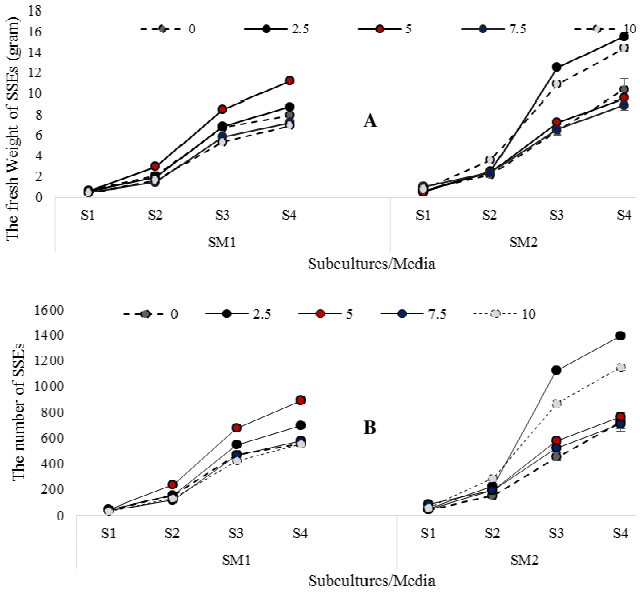

Fig. 1. The effect of selected media and different mT concentrations on (A) fresh weight of SSEs (g) and (B) number of SSEs within the 4 subcultures

SM1: half-strength MS full vitamin; SM2: half-strength MS supplemented with 0.5 mg/L BAP; S1: first subculture; S2: second subculture; S3: third subculture; and S4: fourth subculture

vegetative growth indicator, represented by plant height (cm), (3) number of leaves per plantlet, (4) leaves area (cm^2), (5) number of roots per plantlet, (6) root length (cm), and (7) leaves chlorophyll content (SPAD unit), measured by SPAD meter (SPAD -502, Konica Minolta Co.Ltd, Osaka, Japan) were noted.

Data analysis

Data collected from the experiments was interpreted using analysis of variance (ANOVA) determined with SAS Release Window 9:12. If there were significant effects between treatments, mean values of the treatments were further tested using Duncan multiple range test (DMRT) at *p=0.05* (Mattjik and Sumertajaya, 2006).

Results and Discussion

The effect of selected media and mT on SSE formation

Under periodical observation it was clearly revealed that initiation of SSE was obviously at ± 6 weeks after establishing the culture. The SSE formation was initiated by the emergence of several protrusions in the most surface body of primary embryos, especially in the basal to middle part of them. The protrusions continued to grow and then produced mature SSEs ± 2.0 months after culture establishment. The number of SSEs varied from 4 up to 25 SSEs per primary embryo cultured, thus the result was affected by selected medium and mT concentration.

In the first phase of the experiment, the two selected media and different mT concentrations gave significant different effects on SSE formation; however, there was no significant interaction effect from the treatments, in all parameters observed. Among the two selected media tested, medium SM2 was more suitable to produce a higher number of SSEs.

The medium induced high frequency of SSEs, up to 83.8%, with 12.0 per embryo (Table 1). Even more, 2.5 mg/L was the most appropriated mT concentration in order to stimulate high SSE production, with 64.9% SSE formation and 12.3 SSEs per embryo (Table 2). Saharan *et al.* (2011) reported that the use of 0.1 mg/L mT in combination with 1.0 mg/L 2,4-D in MS medium

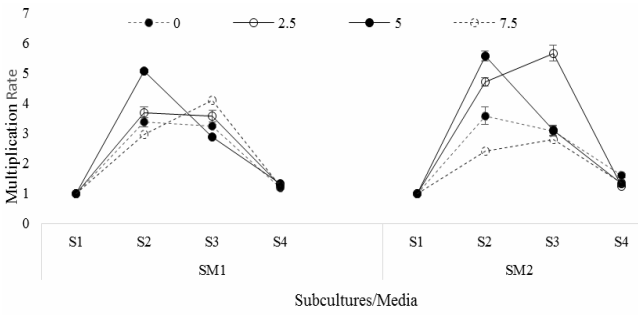

Fig. 2. The effect of selected media and different mT concentrations on SSEs multiplication rate within the 4 subcultures

SM1: half-strength MS full vitamin; SM2: half-strength MS supplemented with 0.5 mg/L BAP; S1: first subculture; S2: second subculture; S3: third subculture; and S4: fourth subculture

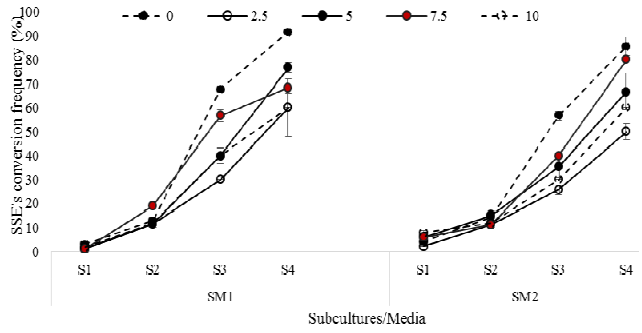

Fig. 3. The effect of selected media and mT concentrations on conversion frequency of SSEs (%) within the 4 subcultures

SM1: half-strength MS full vitamin; SM2: half-strength MS supplemented with 0.5 mg/L BAP; S1: first subculture; S2: second subculture; S3: third subculture; and S4: fourth subculture

Table 3. The effect of mT application on mass propagation and conversion of *Phalaenopsis* somatic embryos within the 4 subcultures

Medium	mT (mg/L)	Total fresh weight of SSE	Total number of SSE	Frequency of SSE conversion (%)	Number of leaves per germinated embryo	Number of roots per germinated embryo
SM1	0.0	7.89 a	552.30 a	91.70 a	1.57 b	1.29 a
	2.5	8.72 a	697.87 a	60.00 a	1.27 b	0.57 ab
	5.0	11.17 a	893.33 a	76.70 a	2.37 ab	0.16 b
	7.5	7.19 a	575.47 a	68.30 a	1.13 b	0.67 ab
	10.0	6.54 a	523.50 a	60.00 a	0.92 b	0.23 b
SM2	0.0	7.36 b	515.00 c	85.70 a	1.31 b	0.97 b
	2.5	15.50 a	1395.30 a	50.00 b	3.59 a	0.31 bc
	5.0	9.55 b	763.90 b	66.70 ab	2.36 ab	0.23 bc
	7.5	8.86 b	708.50 bc	80.00 a	4.30 a	2.82 a
	10.0	14.36 a	1149.10 a	60.00 ab	2.82 ab	0.07 c

SM1: Half-strength MS full vitamin; SM2: Half-strength MS supplemented with 0.5 mg/L BAP. Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test, P=0.05)

Table 4. The effect of media on plantlet growth after 6 weeks on culture

Growth media	Height of plantlet (cm)	Number of leaves per plantlet	Leaves mean area (cm^2)	Number of roots per plantlet	Length of root (cm)
SG1	0.64 a	2.69 a	0.88 b	1.07 b	0.61 b
SG2	0.57 a	2.39 b	1.08 a	1.91 a	1.52 a

SG1: full vitamin half strength MS medium; SG2: half strength MS medium augmented with 1.5 g/L activated charcoal (AC). Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test, P=0.05).

Table 5. The effect of different mT concentration on plantlet growth after 6 weeks on culture

mT concentrations (mg/L)	Height of plantlet (cm)	Number of leaves per plantlet	Leaves mean area (cm^2)	Number of roots per plantlet	Length of root (cm)
0.0	0.48 c	2.48 a	0.96 a	1.90 a	1.06 a
2.5	0.71 a	2.65 a	1.00 a	1.33 b	0.85 a
5.0	0.66 ab	2.44 a	1.05 a	1.40 b	0.80 a
7.5	0.57 abc	2.47 a	0.93 a	1.33 b	0.88 a
10.0	0.62 abc	2.48 a	0.59 a	1.39 b	0.86 a

Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test, P=0.05).

Table 6. The effect of different mT concentration on the growth of plants after 8 weeks on acclimatization

mT concentrations (mg/L)	Survival plants (%)	Plant height (cm)	Number of leaves per plant	Leaves area (cm^2)	Number of roots per plant	Length of roots (cm)	Chlorophyll content (SPAD unit)
0.0	90.00 a	0.70 c	2.50 b	1.22 b	2.75 a	2.34 b	82.15 ab
2.5	86.00 a	0.95 ab	4.00 ab	1.88 a	3.00 a	2.28 b	86.75 a
5.0	80.00 a	0.90 b	4.00 ab	1.68 a	2.83 a	1.60 c	77.87 b
7.5	82.00 a	1.00 a	4.20 a	1.83 a	2.90 a	2.71 b	84.00 ab
10.0	86.00 a	1.02 a	4.00 b	2.04 a	2.90 a	3.63 a	79.84 ab

Means followed by the same letter in the same column are not significantly different (Duncan's multiple range test, P=0.05).

successfully induced a high percentage of SE, up to 68.9%, with maximum number of SEs (98.2 ± 2.3 per g callus culture) in *in vitro* culture of *Balanites aegyptiaca* Del. (L.) derived from root explants. Application of 1.2 mg/L mT in MS medium stimulated high callus formation of *Aloe polyphylla* (Bairu *et al*., 2007), while 0.5 mg/L mT in N6 medium stimulated high embryogenic callus formation for *Citrus clementina* 'Monreal Rosso' derived from microspore with 1.4 embryos per petri dish (Chiancone *et al*., 2015). The results revealed that the higher the concentration of mT, the higher SSE regenerated, but a concentration of mT higher than 2.5 mg/L gradually reduced SSE formation; even so, Meyer *et al*. (2009) reported that the application of 1.3-3.6 mg/L mT in MS medium gave high impact on callus initiation and shoot regeneration of *Hypericum frondosum*.

Effect of selected media and mT concentrations on multiplication and conversion of SSEs

Under periodical subculture of SSEs, it was clearly recorded that fresh weight and number of SSEs produced in each subculture gradually increased (Fig. 1 A, B). However, multiplication rate (MR) of SSEs formation increased in the first and second subculture and decreased afterwards (Fig. 2). The highest MR of SSEs formation, with 10.1 fold in the third subculture and 55% CF, was recorded at 2.5 mg/L mT treatment on SM2 medium. Wojtania (2010) reported that a high multiplication rate of shoot formation (2.7-4.7 fold, depending on genotype) on *Pelargonium* × *hortorum* and *Pelargonium* × *hederaefolium* was noted on MS medium containing 0.5-1.0 mg/L mT. In pineapple, 15.75 fold of multiplication rate of shoot formation was established on MS liquid medium supplemented with 0.6 mg/L mT

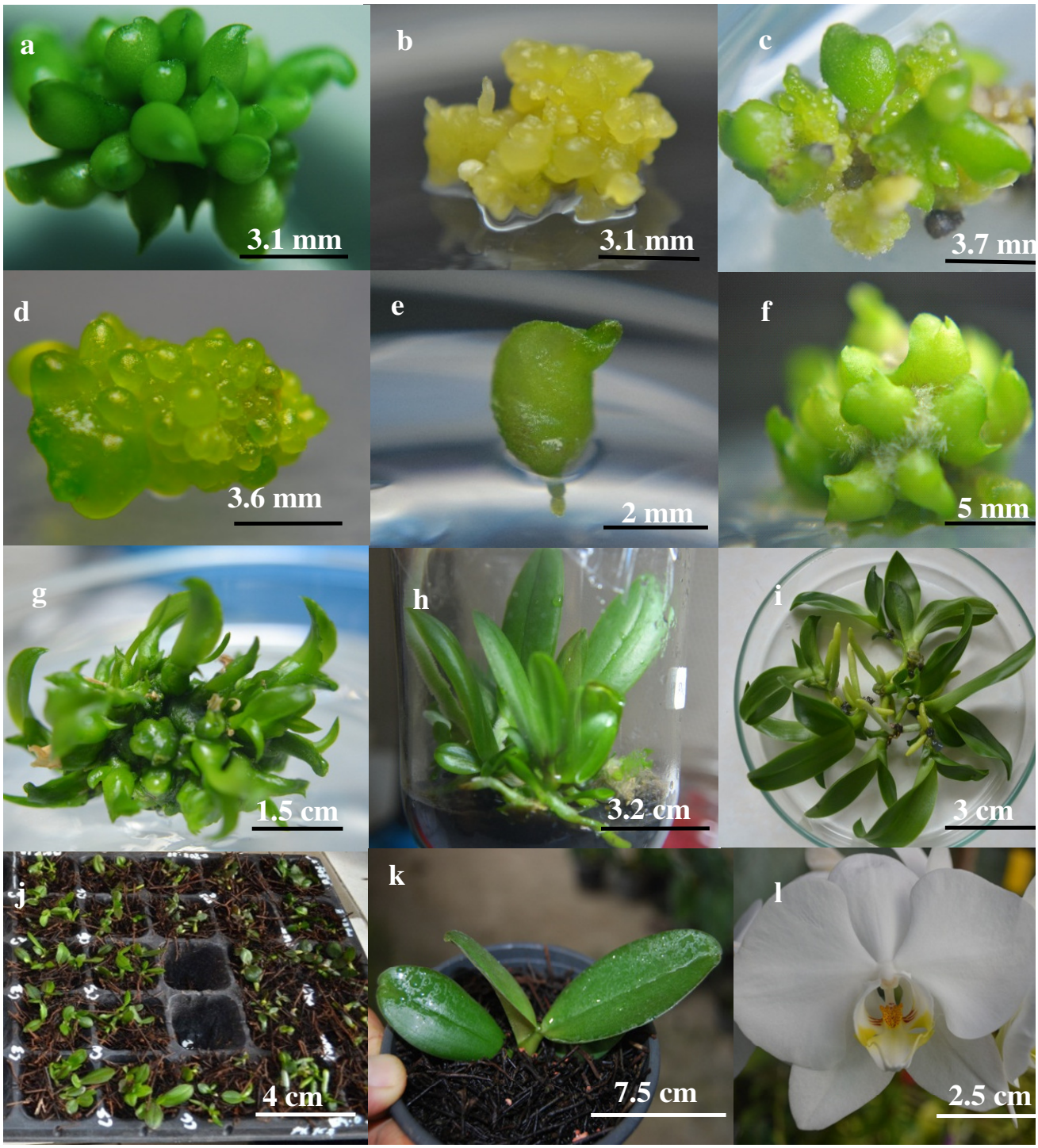

Fig. 4. The effect of meta-topolin (mT) application on propagation of *Phalaenopsis* 'AMP17', via induction of secondary somatic embryos (SSEs). (a) Embryos derived from meristem tip of *Phalaenopsis* 'AMP17' used as explant sources; (b) direct SE from shoot tip culture ± 6 weeks after culture; (c) initiation of SSEs from germinated primary somatic embryos ± 6 weeks after culture; (d) cluster of SSEs on medium containing mT ± 12 weeks after culture; (e) a single converted/germinated embryo; (f) maturation of SSEs on SG2 medium containing 2.5 mg/L mT in initial germination ± 6 weeks after culture; (g) germinated-embryos ± 8 weeks after culture in the similar medium and mT treatment; (h) growth of plantlets on the similar treatment ± 8 weeks after culture; (i) plantlets prepared for acclimatization stage; (j) acclimatized-plantlets on tray with *C. rumphii* bulk; (k) growth of single plant derived from mT treatment 6 months after individually repotting; (l) flower performance of *Phalaenopsis* 'AMP 17'

(Teklehaymanot *et al*., 2010); 12.92 multiplication rate of embryo regeneration of *Paulownia fortunei* on MS medium augmented with 1.0 mg/L mT (Clapa *et al*., 2014).

Effect of mT concentrations on acclimatization of plantlets

Application of different mT concentrations did not gave significant effect on acclimatization of plantlets. All treatments resulted within plantlet survivability percentage of 80- 90%, with the highest percentage of survivability recorded on mT free medium, while application of mT tended to reduce the survivability plantlets during the acclimatization stage.

In *A. polyphylla*, more than 90% of plants treated with mT acclimatized successfully, compared to 65% survival rate recorded with BA treated plants (Bairu *et al*., 2007). Ninety four percent (94%) of *L. meyenii* plantlets were easily acclimatized to *ex vitro* environment in a mixture of peat, soil and sand (Polzerova *et al*., 2011), as well as 96% survival plantlets of *B. aegyptiaca* in a mixture of vermiculite, soil and sand (2:1:1, v/v/v) (Saharan *et al*., 2011).

Though the mT treatments had negative effect on survivability of plantlets, the mT treatments accelerated with significant effect the growth of plants and gave the highest chlorophyll content of 86.75 SPAD unit, as seen in the case of 2.5 mg/L mT treatment (Table 6).

Conclusions

In vitro protocol was successfully established for mass propagation of *Phalaenopsis* 'AMP 17' via secondary embryogenesis. Half-strength MS supplemented with 0.5 mg/L BAP and 2.5 mg/L mT induced high secondary somatic embryo (SSE) formation up to 64.9%, with 12.3 SSEs regenerated per embryo. High multiplication rate of SSE formation up to 10.1 fold, low conversion rate of germinated-embryos as 55% and qualified-growth of the germinated embryos were also recorded in the combination treatment. Survival plantlets, up to 86%, with high qualified-performance were determined in the mT treatment. Establishment of the current protocol may increase the number of high qualified-seedlings obtained and influence their sustainability, in order to propagate *Phalaenopsis* in a commercially efficient manner.

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