In Vitro Propagation and Determination of Total Phenolic Compounds, Flavonoid Contents and Antioxidative Activity of Globba globulifera Gagnep.

Wipa Yaowachai1,3, Surapon Saensouk2,3, Piyaporn Saensouk1,3,*

ABSTRACT

Introduction: Currently, there is a reduction in the number of Globba globulifera, which is due to its slow multiplication rate, high susceptibility to pathogenic diseases and overexploitation of the plant from natural sources. In vitro culture to study suitable concentrations of plant growth regulators for shoot and root induction of G. globulifera. Bioactive compounds were measured by TPC, TFC and FRSA methods for comparison of those from in vitro and natural conditions. Methods: Microshoots were cultured on solid and liquid MS medium supplemented with various concentrations of cytokinins (BA, Kinetin and TDZ) and auxins (NAA and IBA) for eight weeks. Methanol was used as the extraction solvent via the ultrasonic method, TPC and TFC were both measured. DPPH for free radical scavenging activity was investigated. Results: The best result for shoot formation was achieved when culturing on MS medium with 3 mg/l and 5 mg/l of BAP or 5 mg/l of BAP plus 1 mg/l of IBA. The plantlets were transplanted to pots in a greenhouse. All the planting material showed a 100% survival rate. The rhizomes of in vitro-derived plantlets showed the highest value of TPC (52.28 mg GAE/g crude extract) and FRSA (93.65%) and lowest of IC50 (0.46 mg/ml). Conclusion: The in vitro culture and antioxidant activity analysis could be the foundation for plant propagation in large quantities and the use of medicine. Key words: Bioactive compound, DPPH, Micropropagation, TFC, TPC, Zingiberaceae.

INTRODUCTION

The genus Globba is the third most diverse of herbaceous plants in the tribe Globbeae, family Zingiberaceae. Globba is distributed in tropical regions of Asia, including across all regions of Thailand. The floral structure of the plants in the genus Globba is like a dancing woman, so it is commonly known as dancing lady ginger. Many species in the genus Globba are used economically, such as G. schomburgkii and G. adhaerens, which are used as ornamental plants and for export. Some species are used as herbs, such as the roots of G. leucantha and G. patens that are boiled for use by women after childbirth. Moreover, an extract from the leaves of G. marantina is used as eye drops to treat conjunctivitis and eye inflammation. The essential oil of G. winitii can inhibit the bacteria Staphylococcus aureus, Proteus mirabilis and Micrococcus luteus.1

Globba globulifera is a herbaceous plant in the genus Globba with a height of 30-50 cm. The inflorescences have dark purple petal-like structures, which are called bracts, with yellow and small flowers. Therefore, it is used as an ornamental plant. The rhizomes and leaves contain essential oils. The flowering occurs from May to September, and dormancy in the winter. G. globulifera is propagated by rhizomes, seeds and bulbil, which are both measured. Methanol was used as the extraction solvent via the ultrasonic method, TPC and TFC were both measured. DPPH for free radical scavenging activity was investigated. Results: The best result for shoot formation was achieved when culturing on MS medium with 3 mg/l and 5 mg/l of BAP or 5 mg/l of BAP plus 1 mg/l of IBA. The plantlets were transplanted to pots in a greenhouse. All the planting material showed a 100% survival rate. The rhizomes of in vitro-derived plantlets showed the highest value of TPC (52.28 mg GAE/g crude extract) and FRSA (93.65%) and lowest of IC50 (0.46 mg/ml). Conclusion: The in vitro culture and antioxidant activity analysis could be the foundation for plant propagation in large quantities and the use of medicine. Key words: Bioactive compound, DPPH, Micropropagation, TFC, TPC, Zingiberaceae.

MATERIALS AND METHODS

In vitro shoot regeneration

Surface-sterilization procedures and initial culture establishment

The bulbils of G. globulifera from plants growing under a natural environment were collected from Udorn Thani Province, Thailand. The bulbils were washed for 30 min under running tap water, soaked for 1 min in 70% (v/v) ethanol, followed by
disinfection with 20% and 15% sodium hypochlorite (NaOCl) for 20 min and 15 min under aseptic conditions, respectively. Then, rinsed in sterilized distilled water three times for 5 min. Initially, the bulbils were grown on MS medium (Murashige and Skoog) supplement with a combination of 2.0 mg/l BAP with 1.0 mg/l NAA.

**Shoot multiplication**

The shoot explants were transferred to solid MS medium supplemented with various concentrations of plant growth regulators. The plant growth regulators were BAP (0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l), Kinetin (0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l), BAP (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) in combination with NAA (0.5 and 1 mg/l), BAP (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) in combination with IBA (0.5 and 1 mg/l), Kinetin (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) in combination with NAA (0.5 and 1 mg/l) and Kinetin (1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) in combination with IBA (0.5 and 1 mg/l). The MS liquid medium supplemented with 3.0 mg/l of Kinetin in combination with 0.5 mg/l of NAA (Parida et al.)², 2.0 mg/l of BAP (Chuenpengaya et al.³) and 2.0 mg/l of TDZ (Saensouk et al.) was used for the culture media. The explants were cultured on liquid and solid (7 g/l of bacto agar) MS media to study the effect of the type of media. For all media, the pH was modified to 5.7–5.8 and the media was autoclaved at 121 °C for 15 min, with 10 replications for each procedure. Mean number of shoots, mean length of shoot (cm), mean number of roots and mean length of root (cm) per explant were measured. Cultures were incubated with fluorescent lamps at 25 ± 2 °C under a 16 h photoperiod for eight weeks.

**Acclimatization**

The plantlet was kept at room temperature for four weeks and then after culturing in the MS medium for another eight weeks. The plantlets had shoots and roots that were well established, and they were 6-8 cm in height. Then the plantlets were removed from the glass bottle. The roots were gently washed in running tap water to remove the crop media debris and transferred to the greenhouse for eight weeks in plastic pots containing soil, sand and soil mixed with sand (1:1 w/w). The survival rate, mean number of shoots, mean length of shoot (cm), mean number of leaves, mean length of leaf (cm) and mean width of leaf (cm) per plantlet were measured for eight weeks.

**Bioactive Compounds**

**Plant material**

*In vitro* derived plantlets for one year and those derived from natural conditions were used as the plant material. The parts of *G. globulifera* used in the study were the rhizomes, root storage, leaves and pseudostems. Healthy plants were selected, wash thoroughly with running tap water and cut into small pieces. The pieces of *G. globulifera* were first frozen for 12 hours at -80 °C, and then quickly put into a freeze dryer. Ground thoroughly with a herb grinder, and then the specimens were stored in a desiccant jar.

**Antioxidant extraction**

Antioxidant extraction was according to the procedure described by An et al. with modifications. For this, 5 g of *G. globulifera* explant powder was precisely weighed, then added to 30 ml 80% methanol. Each different explant was extracted ultrasonically for 30 min three times and filtered. The methanol was removed in a freeze dryer through drying. The extraction procedure was repeated three times. The sample solution was prepared from the crude extract. For this, 1 mg of the crude extract was added into the microcentrifuge tube, and then 1 ml of 80% methanol was added and mixed with a vortex (1 mg/ml stock solution).

**Determination of total phenolic contents (TPC)**

The total phenolic contents in the extracts was determined using the Folin–Ciocalteu assay as describe by Lister et al. with modification. Samples (100 µl) were added to a microcentrifuge tube that contained 500 µl of 10% (v/v) Folin–Ciocalteu phenol reagents. They were mixed and incubated in a dark place for 1 min. Then a solution of 400 µl of 7.5% (w/v) sodium carbonate was added to the mixture, and then placed in the dark for 30 min. Then 80% (v/v) methanol was used as a blank. The absorbance was measured at 765 nm using a spectrophotometer. Gallic acid (3-100 mg/l) was used as a standard. The total phenolic content was expressed as mg gallic acid equivalent per gram of crude extract (mg GAE/g crude extract).

**Determination of total flavonoid contents (TFC)**

TFC in the extract was determined using the Aluminium trichloride method as describe by Zhishen et al. with modification. Initially, 50 µl of each sample was added to a microcentrifuge tube. Then 30 µl of 5% (w/v) NaNO₂ was added to the mixture, and after 6 min it was placed in the dark, 60 µl of 10% (w/v) AlCl₃ was added. After 6 min in the dark, and 300 µl of 1 M NaOH was added. Following this, 560 µl of deionize water was added to the reaction and mixed well. Then 80% (v/v) methanol was used as a blank. The absorbance was measured at 510 nm using a spectrophotometer. Catechin (3-200 mg/l) was used as the standard. TFC was expressed as mg catechin equivalent per gram of crude extract (mg CE/g crude extract).

**Determination of DPPH radical scavenging assay**

2,2-Diphenyl-1-picrylhydrazyl (DPPH), which is stable, was used to determine the free radical scavenging capacity as described by Yan and Hsieh with modification. For this, 335 µl of a range of dilutions of the extracts (0.05-1 mg/ml) was added to 665 µl of 0.2 mM DPPH solution in methanol and mixed well, and after this it was incubated at room temperature in the dark for 30 min. The absorbance was measured at 510 nm using a spectrophotometer. The absorbance was used to calculate the free radical scavenging activity (FRSA, %) with the equation: %FRSA = [(A sample - A sample)/A standard] x 100. The half maximal inhibitory concentration (IC₅₀) was determined from the plotted graph of %FRSA and scavenging activity against the concentration of the sample extracts. The scavenging ability of the antioxidants by the IC₅₀ value of each sample was compared with the IC₅₀ values of ascorbic acid.

**Statistical analysis**

The experiments were conducted using a completely randomized design (CRD). Data were analyzed for significance using ANOVA and differences were contrasted using a Duncan’s multiple range test (p<0.05) with the SPSS program version 15.

**RESULTS**

**In vitro shoot regeneration**

Microshoots of *G. globulifera* (1 cm length) were cultured on MS solid medium for eight weeks with various concentrations of a plant growth regulator for shoot and root formation. The highest number of shoots was obtained on the medium supplemented with 3 mg/l and 5 mg/l of BAP (4.40 shoots/explant). The best shoot length was observed on 3 mg/l of BAP (2.67 cm). The highest average number of roots per shoot and the best root length were observed in 5 mg/l and 4 mg/l of BAP, respectively (24.20 roots/explant and 1.67 cm) (Table 1 and Figure 1). The MS medium containing 5 mg/l of Kinetin showed the highest number of shoots (4.80 shoots/explant), shoot length (2.42 cm), average number of roots (23.20 roots/explant) and root length (2.26 cm) (Table 2 and Figure 1).
The effect of a combination of cytokinins (BA and Kinetin) and auxins (NAA and IBA) showed well-developed shoot and root formation. MS medium supplemented with BAP combination with NAA or IBA had suitable shoot and root multiplication. The highest number of shoots was observed on the medium containing 5 mg/l of BAP plus 1 mg/l of IBA (6.40 shoots/explant) and the best shoot length was found on medium supplemented with 4 mg/l of BAP plus 0.5 mg/l of NAA (2.68 cm). The highest average number of roots and the best root length was observed in 2 mg/l of BAP plus 0.5 mg/l of IBA (34.50 roots/explant) and 5 mg/l of BAP plus 0.5 mg/l of NAA (1.99 cm), respectively (Table 3). The MS medium supplement with 5 mg/l of Kinetin in combination with 1 mg/l of NAA showed the highest number of shoots (6.50 shoots/explant), shoot length (3.20 cm) and average number of roots (46.50 roots/explant) (Table 4). There were statistically significant differences when comparing the different plant growth regulators on liquid media. The liquid MS medium supplemented with 2.00 mg/l of TDZ show the highest number of shoots (12.25 shoots/explant) (Table 5 and Figure 2).

### Table 1: Effect of BAP on shoot and root multiplication of *G. globulifera* for eight weeks.

| BAP (mg/l) | Average no. of shoots/explant mean ± SE | Average shoot length (cm) mean ± SE | Average no. of roots/explant mean ± SE | Average root length (cm) mean ± SE |
|------------|----------------------------------------|------------------------------------|---------------------------------------|-----------------------------------|
| 0          | 2.60 ± 0.22ab                         | 1.07 ± 0.06a                        | 11.90 ± 1.14a                          | 0.69 ± 0.04d                      |
| 1          | 3.10 ± 0.23ac                         | 1.36 ± 0.03d                        | 13.20 ± 0.55d                          | 1.20 ± 0.04e                      |
| 2          | 3.50 ± 0.27bc                         | 1.52 ± 0.05d                        | 20.00 ± 0.49a                          | 1.21 ± 0.02b                      |
| 3          | 4.40 ± 0.22ab                         | 2.67 ± 0.10d                        | 21.20 ± 0.79a                          | 1.33 ± 0.05c                      |
| 4          | 4.10 ± 0.28a                        | 2.10 ± 0.06d                        | 22.80 ± 0.59a                          | 1.67 ± 0.03e                      |
| 5          | 4.40 ± 0.31a                        | 2.38 ± 0.09d                        | 24.20 ± 0.53a                          | 1.50 ± 0.07b                      |

Means followed by same letters within each column are not significantly different at p≤0.05, according to DMRT.

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### Table 2: Effect of Kinetin on shoot and root multiplication of *G. globulifera* for eight weeks.

| Kinetin (mg/l) | Average no. of shoots/explant mean ± SE | Average shoot length (cm) mean ± SE | Average no. of roots/explant mean ± SE | Average root length (cm) mean ± SE |
|---------------|----------------------------------------|------------------------------------|---------------------------------------|-----------------------------------|
| 0             | 2.60 ± 0.22c                           | 1.07 ± 0.06c                        | 11.90 ± 1.14c                          | 0.69 ± 0.04c                      |
| 1             | 2.80 ± 0.25e                           | 2.11 ± 0.15d                        | 19.60 ± 0.50c                          | 1.52 ± 0.11c                      |
| 2             | 3.80 ± 0.25e                           | 1.96 ± 0.09f                        | 18.70 ± 0.67c                          | 1.77 ± 0.12f                      |
| 3             | 3.90 ± 0.28e                           | 1.95 ± 0.05g                        | 21.30 ± 0.73a                          | 1.42 ± 0.06f                      |
| 4             | 3.80 ± 0.29e                           | 2.23 ± 0.13a                        | 22.70 ± 0.67a                          | 1.95 ± 0.07f                      |
| 5             | 4.80 ± 0.29a                           | 2.42 ± 0.09a                        | 23.20 ± 0.79a                          | 2.26 ± 0.09a                      |

Means followed by same letters within each column are not significantly different at p ≤ 0.05, according to DMRT.

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**Figure 1:** Multiple shoot formation of *G. globulifera* after eight weeks of cultivation on MS medium supplemented with plant growth regulators. (A. MS (control), B. BAP 3 mg/l, C. BAP 5 mg/l, D. Kinetin 5 mg/l, E. BAP 5 mg/l + IBA 1 mg/l and F. Kinetin 5 mg/l + NAA 1 mg/l).
Means followed by same letters within each column are not significantly different at $p \leq 0.05$, according to DMRT.

### Table 4: Effect of combination with Kinetin plus NAA and Kinetin plus IBA on shoot and root multiplication of *G. globulifera* for eight weeks.

| Kinetin (mg/l) | NAA (mg/l) | IBA (mg/l) | Average no. of shoots/explant mean ± SE | Average shoot length (cm) mean ± SE | Average no. of roots/explant mean ± SE | Average root length (cm) mean ± SE |
|---------------|------------|------------|----------------------------------------|-----------------------------------|----------------------------------------|-----------------------------------|
| 0             | 0          | 0          | 2.60 ± 0.22$^{b}$                       | 1.07 ± 0.06$^{d}$                 | 11.90 ± 1.14$^{e}$                     | 0.69 ± 0.04$^{d}$                 |
| 1             | 0.5        | 0          | 4.60 ± 0.27$^{d}$                       | 2.09 ± 0.11$^{c}$                 | 22.60 ± 1.61$^{d}$                     | 1.87 ± 0.17$^{d}$                 |
| 2             | 0.5        | 0          | 4.00 ± 0.42$^{d}$                       | 2.12 ± 0.15$^{d}$                 | 21.50 ± 2.83$^{d}$                     | 1.68 ± 0.13$^{d}$                 |
| 3             | 0.5        | 0          | 4.70 ± 0.40$^{d}$                       | 1.96 ± 0.12$^{d}$                 | 20.70 ± 1.96$^{d}$                     | 1.72 ± 0.12$^{d}$                 |
| 4             | 0.5        | 0          | 5.00 ± 0.26$^{d}$                       | 2.68 ± 0.21$^{d}$                 | 24.00 ± 2.17$^{d}$                     | 1.92 ± 0.11$^{d}$                 |
| 5             | 0          | 0          | 4.40 ± 0.27$^{d}$                       | 2.25 ± 0.17$^{d}$                 | 23.00 ± 1.53$^{d}$                     | 1.99 ± 0.13$^{d}$                 |
| 1             | 1          | 0          | 5.90 ± 0.31$^{d}$                       | 2.38 ± 0.22$^{d}$                 | 27.70 ± 1.69$^{d}$                     | 1.97 ± 0.15$^{d}$                 |
| 2             | 1          | 0          | 4.80 ± 0.47$^{d}$                       | 2.34 ± 0.23$^{d}$                 | 29.20 ± 1.87$^{d}$                     | 1.82 ± 0.16$^{d}$                 |
| 3             | 1          | 0          | 4.10 ± 0.31$^{d}$                       | 2.28 ± 0.25$^{d}$                 | 24.90 ± 2.48$^{d}$                     | 1.92 ± 0.25$^{d}$                 |
| 1             | 1          | 0.5        | 4.70 ± 0.15$^{d}$                       | 1.84 ± 0.16$^{d}$                 | 23.50 ± 2.21$^{d}$                     | 1.40 ± 0.09$^{d}$                 |
| 5             | 1          | 0          | 4.40 ± 0.43$^{d}$                       | 1.67 ± 0.10$^{d}$                 | 17.60 ± 3.19$^{d}$                     | 1.38 ± 0.21$^{d}$                 |
| 1             | 0.5        | 0          | 4.30 ± 0.42$^{d}$                       | 1.68 ± 0.14$^{d}$                 | 29.50 ± 2.80$^{d}$                     | 1.10 ± 0.05$^{d}$                 |
| 2             | 0.5        | 0          | 4.60 ± 0.50$^{d}$                       | 1.87 ± 0.19$^{d}$                 | 34.50 ± 2.63$^{d}$                     | 1.33 ± 0.18$^{d}$                 |
| 3             | 0.5        | 0          | 4.80 ± 0.73$^{d}$                       | 2.04 ± 0.24$^{d}$                 | 33.70 ± 6.65$^{e}$                     | 1.56 ± 0.20$^{d}$                 |
| 4             | 0.5        | 0          | 4.50 ± 0.34$^{d}$                       | 1.70 ± 0.13$^{d}$                 | 18.50 ± 1.98$^{d}$                     | 1.35 ± 0.12$^{d}$                 |
| 5             | 0.5        | 0          | 5.00 ± 0.21$^{d}$                       | 1.69 ± 0.09$^{d}$                 | 22.90 ± 0.86$^{d}$                     | 1.37 ± 0.04$^{d}$                 |
| 1             | 0          | 1          | 3.80 ± 0.49$^{d}$                       | 1.60 ± 0.11$^{d}$                 | 26.80 ± 2.57$^{d}$                     | 1.62 ± 0.27$^{d}$                 |
| 2             | 0          | 1          | 3.60 ± 0.37$^{d}$                       | 1.51 ± 0.17$^{d}$                 | 21.90 ± 2.01$^{d}$                     | 1.17 ± 0.09$^{d}$                 |
| 3             | 0          | 1          | 5.20 ± 0.77$^{d}$                       | 1.43 ± 0.11$^{d}$                 | 21.40 ± 3.39$^{d}$                     | 1.22 ± 0.07$^{d}$                 |
| 4             | 0          | 1.5        | 5.90 ± 0.64$^{d}$                       | 1.60 ± 0.13$^{d}$                 | 25.10 ± 2.76$^{d}$                     | 1.33 ± 0.18$^{d}$                 |
| 5             | 0          | 1          | 6.40 ± 0.70$^{d}$                       | 2.05 ± 0.26$^{d}$                 | 26.00 ± 2.45$^{d}$                     | 1.35 ± 0.09$^{d}$                 |

Means followed by same letters within each column are not significantly different at $p \leq 0.05$, according to DMRT.

### Table 5: Effect of liquid medium supplemented with Kinetin, NAA, BAP and TDZ on shoot and root multiplication of *G. globulifera* for eight weeks.

| Kinetin (mg/l) | NAA (mg/l) | BAP (mg/l) | TDZ (mg/l) | Average no. of shoots/explant mean ± SE | Average shoot length (cm) mean ± SE | Average no. of roots/explant mean ± SE | Average root length (cm) mean ± SE |
|---------------|------------|------------|------------|----------------------------------------|-----------------------------------|----------------------------------------|-----------------------------------|
| 0             | 0          | 0          | 0          | 3.67 ± 0.19$^{d}$                       | 2.25 ± 0.19$^{d}$                 | 12.08 ± 1.09$^{d}$                     | 1.10 ± 0.07$^{d}$                 |
| 3             | 0.5        | 0          | 0          | 3.75 ± 0.28$^{d}$                       | 1.97 ± 0.14$^{d}$                 | 8.58 ± 1.14$^{d}$                      | 1.27 ± 0.08$^{d}$                 |
| 0             | 0          | 2          | 0          | 4.92 ± 0.45$^{d}$                       | 2.84 ± 0.18$^{d}$                 | 20.92 ± 1.54$^{d}$                     | 1.51 ± 0.05$^{d}$                 |
| 0             | 0          | 0          | 2          | 12.25 ± 0.88$^{d}$                      | 2.00 ± 0.16$^{d}$                 | 4.08 ± 0.58$^{d}$                      | 0.76 ± 0.11$^{c}$                 |

Means followed by same letters within each column are not significantly different at $p \leq 0.05$, according to DMRT.
Plantlets (6-8 cm in height) with well-developed shoots and roots were transferred for eight weeks during the rainy season into a greenhouse at the Department of Biology, Faculty of Science, Mahasakham University, Mahasarakham, Thailand. After eight weeks, plantlets of *G. globulifera* transplanted into the soil, sand and soil mixed with sand produced various sizes of storage roots and new leaves. They are well adapted to the external environment and can be used as planting material. All the planting material showed 100% survival rate. The maximum number of shoots (4.35 shots/plant), average shoot length (14.03 cm) and number of leaves (31.55 leaves/plant) were observed in soil (Table 6 and Figure 3).

### Bioactive Compounds

Antioxidant activity is an excellent example of how plant extracts can provide a practical gain.\(^{10}\) From the results of TPC, TFC and free radical scavenging activity (FRSA) of methanol extracts of *ex vitro* plantlets and *in vitro*-derived plantlets, the results specified that there is a significant difference between the extracts of different parts of the plantlets. The TPC demonstrated in gallic acid equivalents showed the highest value of 52.28 mg GAE/g crude extract in the rhizome of *in vitro*-derived plantlets, whereas the rhizome of *ex vitro* plantlets showed 50.56 mg GAE/g crude extract (Table 7). Likewise, the rhizome of the *in vitro*-derived plantlets had the highest FRSA (93.55%) and lowest IC\(_{50}\) (0.46 mg/ml). TFC was expressed in catechin equivalents and showed the highest value of 33.62 mg CE/g crude extract in the rhizome of *ex vitro* plantlets. The DPPH scavenging ability had higher correlations with TPC (*r* = 0.913), and less correlation with TFC (*r* = 0.894).

### DISCUSSION

When the microshoots of *G. globulifera* were cultured on the MS medium with various concentrations of BAP or Kinetin alone, BAP plus NAA or IBA, Kinetin plus NAA or IBA added, the shoot and root formation were different. In the same genus, the BAP treatments are consistent with previous studies.\(^{12}\)

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**Figure 2:** Multiple shoot formation of *G. globulifera* after eight weeks of cultivation on liquid MS medium supplemented with plant growth regulators. (A. MS (control), B. Kinetin 3 mg/l + NAA 0.5 mg/l, C. BAP 2 mg/l and D. TDZ 2 mg/l).

**Figure 3:** Acclimatized plantlets eight weeks after transfer to pots in a greenhouse. (A. soil, B. sand and C. soil:sand (1:1)).

**Table 6:** Effect of potting media on plantlet performance of *G. globulifera* after eight weeks of acclimatization.

| Potting medium          | Average no. of shoots/plant mean ± SE | Average shoot length (cm) mean ± SE | Average no. of leaves/plant Mean ± SE | Survival rate (%) |
|-------------------------|--------------------------------------|------------------------------------|---------------------------------------|-------------------|
| Soil                    | 4.35 ± 0.42\(^a\)                   | 14.03 ± 0.51\(^a\)                | 31.55 ± 2.19\(^a\)                   | 100               |
| Sand                    | 2.90 ± 0.2\(^b\)                    | 6.27 ± 0.37\(^b\)                 | 14.80 ± 1.11\(^b\)                   | 100               |
| Soil mixed with sand    | 3.55 ± 0.27\(^b\)                   | 7.23 ± 0.58\(^b\)                 | 19.90 ± 1.46\(^b\)                   | 100               |

Means followed by same letters within each column are not significantly different at *p*≤0.05, according to DMRT.
with Kho et al.\textsuperscript{11} who reported the medium supplement with 3 mg/l of BAP showed the highest number of shoots (6.60 shoots/explant) when culturing G. bracasyanthera. Nevertheless, these results differed from those of Chuengpanya et al.\textsuperscript{4} who reported that the maximum number of shoots was 24.1 shoots/explant and 180.4 roots/explant when culturing G. siamensis on MS medium containing 2.0 mg/l of BAP. Saensouk et al.\textsuperscript{1} reported different results, the MS medium supplemented with 3.0 mg/l of BAP show the number of shoots was 3.80 shoots/explant of G. schombergkii. Then, 5 mg/l of Kinetin showed the best response on shoot and root induction. These results are different from Parida et al.\textsuperscript{12} who reported the maximum number of shoots was 5.8 shoots/explant when culturing G. marantina on a medium that contained 3 mg/l of Kinetin. The result of comparing different plant growth regulators on liquid media showed the differences from Saensouk et al.\textsuperscript{1} who reported in \textit{in vitro} propagation of G. schombergkii on MS medium supplemented with 2.00 mg/l of TDZ (9.10 shoots/explant).

A culture medium was supplemented with cytokinins (BAP, Kinetin and TDZ) displayed a strong effect on the proliferation rate of the micropropagation process. In general, BAP, Kinetin and TDZ hormones have been the most commonly used media and plant growth regulators in plant micropropagation. Cytokinin also mediates the responses to irrelevant factors, such as light conditions in the shoot and accessibility of nutrients and water in the root.\textsuperscript{13} BAP could increase the level of cytokinin in plants, protects the pith tissue and prolongs the active photosynthetic duration and improves the photosynthetic efficiency factors.\textsuperscript{14} Kinetin could maintain the stability of chlorophyll and enhances the activities of antioxidant enzymes.\textsuperscript{15} TDZ could be an effective bioregulator in plant tissue cultures in a wide array of plant species.\textsuperscript{16}

In the present study, it was found that using a plant growth regulator combination (BAP plus NAA or IBA and Kinetin plus NAA or IBA) could induce shoot formation in similar amounts. The results show the best shoot and root induction occurred on the medium supplement with BAP plus NAA or IBA. These results are different from Parida et al.\textsuperscript{12} who studied the effect of combinations of plant grow regulators. MS medium supplement with 3 mg/l of BAP plus 0.5 mg/l of NAA showed the best number of shoots (6.5 shoots/explant). The effect of Kinetin in combination with NAA or IBA demonstrated an increase in the number of plantlets as well. In the same family, these results are consistent with Nguyen et al.\textsuperscript{17} who reported a medium supplement with 1 mg/l of Kinetin plus 0.5 mg/l of NAA have the highest number of shoots (5.10 shoots/explant) and best shoot length (6.43 cm). However, root induction was effective when using a combination of cytokinin and auxin. NAA is a plant growth regulator in the auxin group and is used for plant tissue culture. It is also a rooting agent and used for vegetative propagation of plants from stem and leaf explants.\textsuperscript{18} The number of shoots per explant induced depended on the ratio of cytokinin and auxin used for shoot multiplication. Cytokinins are responsible for the regulation of plant growth through an influence on the number or duration of cell division cycles in the root and shoot meristems.\textsuperscript{19} Auxins play a major role in root development, apical dominance and plant senescence, and is associated with cell expansion, cell division and cell differentiation.\textsuperscript{20}

In the present study, \textit{in vitro} plantlets derived using the tissue culture technique produced new shoots, roots and leaves. The plantlet was healthy and morphologically like the mother plants after being transferred to the field. These results differ from those of Saensouk et al.\textsuperscript{1} who reported that when the plantlets of G. schombergkii were transferred to pots containing sand they showed the highest percentage of surviving plantlets (80%), average number of leaves per shoot (21.62) and average shoot length (15.00 cm). In this study, \textit{in vitro} plantlets derived from tissue culture techniques produced healthy and plants morphologically similar to the mother after being transferred to the field.

Plants contain a wide range of bioactive compounds with essential health and food applications. Among these compounds, a strong correlation was observed between antioxidant activity and the total plant phenolic content, indicating that phenolic compounds may be the main contributor to their antioxidant ability.\textsuperscript{21} The characterization of the phytochemical contents of different tissues of \textit{G. globulifera} is critical to establishing its medicinal importance. The total phenol and flavonoid contents of \textit{in vitro}-derived plantlets for one year and natural conditions are the rhizomes, storage root, leaves and pseudostems. In this study, it was demonstrated that \textit{in vitro}-derived plantlets had higher levels of phenolic and antioxidant activities than those in natural plantlets. This is consistent with Shohael et al.\textsuperscript{22} who reported that in \textit{in vitro} conditions enhanced the secondary production of metabolites through the modulation of primary plant metabolism. The rate of synthesis and gene expression of secondary metabolites may be significantly affected by the nutritional content and plant hormones used in the \textit{in vitro} culture.\textsuperscript{23}

**CONCLUSION**

In conclusion, a wide-ranging investigation into \textit{in vitro} propagation by shoot proliferation, measurement of the total phenolic contents, total flavonoid contents and the free radical scavenging activity (FRSA) analysis, was conducted. Consequently, the present research will contribute to commercial production and pharmacologically significant conservation. The determination of the free radical scavenging potential of methanol extracts from \textit{G. globulifera} can be used for therapeutic benefits. This study presents the first report of shoot multiplication, acclimatization, total phenolic contents, total flavonoid contents and...
antioxidant activity of *G. globulifera*. This significant study could be useful for propagating *G. globulifera* at a commercial scale, as well as for genetic improvement of this valuable plant in the future.

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GRAPHICAL ABSTRACT

ABOUT AUTHORS

Wipa Yaowachai is a student studying for a M.Sc. degree in Biology at Department of Biology, Faculty of Science, Mahasarakham University, Thailand. Her research projects focus on Plant Tissue Culture, Plant extraction and Bioactive compounds.

Surapon Saensouk is an Associate Professor and lecturer at Walairukhavej Botanical Research Institute, Mahasarakham University, Thailand. His research projects focus on Plant Taxonomy, Biodiversity, Chromosome, Pollen, Plant Anatomy, Plant Geography and Ethnobotany.

Piyaporn Saensouk is an Assistant Professor and lecturer at Department of Biology, Faculty of Science, Mahasarakham University, Thailand. Her research projects focus on Plant Tissue Culture, Plant Anatomy, Palynology and Plant Chromosome.

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