In Vitro Propagation by Asymbiotic Seed Germination and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity Studies of Tissue Culture Rais ed Plants of Three Medicinally Important Species of Dendrobium

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A simple and efficient plant propagation system has been developed by asymbiotic germination of seeds in three medicinally important Dendrobium species, namely, Dendrobium tosae nae, Dendrobium moniliforme, and Dendrobium linawianum. Plants obtained from natural habitats were grown in the greenhouse. The flowers were hand pollinated. Seeds of the capsules derived after 12 weeks of hand-pollination germinated asymbiotically (50–74%) on half strength Murashige and Skoog’s (MS) basal medium with 3% sucrose and solidified with 0.9% Difco agar. Active growth in the germinated seedlings was achieved by re-culturing on full strength MS basal medium supplemented with 8% banana homogenate, 8% potato homogenate, 8% coconut water, 1.5% sucrose and 0.9% Difco agar. Healthy plantlets, transferred to plastic trays containing moss or moss and tree fern, successfully acclimatized (84–100%) in the greenhouse. A marked varied response was observed in the free radical scavenging activity of methanolic extracts of in vitro propagated plants, on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical using a UV spectrophotometer assay. Methanolic extracts were prepared by dissolving the powdered plant material, obtained from six months old in vitro propagated plants, each about 5 g, in boiling methanol. The percentage of scavenging effect of D. tosae nae extract was 95.9% at 0.4 mg/ml concentration, whereas D. moniliforme, and D. linawianum extracts scavenged 83.4% and 92.3%, respectively, at a concentration of 0.4 mg/ml. All the extracts scavenged DPPH radical significantly in a concentration dependent manner.

Key words antioxidant; asymbiotic seed germination; Dendrobium; free radical scavenging activity; mass propagation

The genus Dendrobium (Orchidaceae) includes about 1600 species in the world,1) of which are found in Taiwan.2) Its major distribution regions extend from Japan, Korea, China, and Taiwan through the Indo–Malayan region and Indonesia to New Guinea and Australia. The stems of several Dendrobium species are used in traditional Chinese medicine as a Yin tonic to nourish the stomach, promote the digestion of food, reduce fever, and treat diabetes, and carcinogenesis, are well documented.22–25) Atherosclerosis, skin aging, nephrites, reperfusion injury, asthma, diabetes, and carcinogenesis, are well documented.26) In recent years, therefore, the search for natural antioxidants and other preparations of plant origin to achieve this objective has been intensified. Many Chinese medicinal herbs are reported to possess antioxidative free radical scavenging, mutagenic and antimutagenic activities.27) Several methods have been developed to measure the free radical scavenging capacity, regardless of the individual compounds, which contribute towards the total capacity of a plant product in scavenging free radicals. The methods are typically based on the inhibition of the accumulation of oxidized products, since the generation of free radical species is inhibited by the addition of antioxidants, and this gives rise to a reduction of the end point by scavenging free radicals. A reliable method to determine radical scavenging capacity involves measurement of the disappearance of free radicals, such as 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic) acid radical (ABTS+•), the 1,1-diphenyl-2-picrylhydrazyl (DPPH) or other colored radicals, with a UV spectrophotometer.28,29)

The aims of the present investigations are 1) to develop an efficient and simple tissue culture method for obtaining healthy plants via asymbiotic germination of the immature seeds in the three Dendrobium species, namely, D. tosae nae, D. moniliforme, and D. linawianum; and 2) to investigate the free radical scavenging activity of methanolic extracts of in vitro propagated plants grown in the greenhouse. To our knowledge, no previous investigations have been done on the radical scavenging effects of the species of the genus Dendrobium using the DPPH method.

MATERIALS AND METHODS

Plant Materials and Pollination Mature plants of the

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three *Dendrobium* species were collected from various places in Taiwan: *D. linawanum* from Wu Lai in the month of April 1999, *D. monoliforme* from Chi Lan in the month of March 2000, and *D. tosaense* from Tien Hsiang in the month of March 2000. The healthy plants were replanted in pots (diameter 13.5 cm×height 10.7 cm) containing the substrate tree fern. The plants were maintained in a greenhouse under natural light, 70% relative humidity, and 25/20 °C day/night temperature until flowering. A voucher specimen of each plant species has been deposited at the China Medical College herbarium, Taichung, Taiwan. *Dendrobium tosaense* MAKINO CMC DT 0303; *Dendrobium monoliforme* Sw CMC DM 0302; and *Dendrobium linawanum* REICH. f. CMC DL.0301.

For pollination, the pollen from fully opened flower's pollinia was picked up using forceps and deposited on a stigma of another flower on a different plant. The hand-pollinated flowers were individually marked with tags. After pollinating the plants, the capsules were allowed to ripen by maintaining plants in the greenhouse for 12 weeks.

**Seed Culture and Germination** Capsules collected from hand-pollinated flowers after 12 weeks of pollination were surface-disinfected in 70% ethanol for 30 s, followed by 1.0% sodium hypochlorite (Clorox, The Clorox Co., Oakland, CA, U.S.A.) with two drops of Tween 20® per 100 ml (Hayashi Pure Chemical Industries Ltd., Osaka, Japan) under ultrasonic vibration (Branson Ultrasonic Cleaner, Branson Cleaning Equipment Co., Shelton, CT, U.S.A.) for 10 min, then rinsed five times with sterile distilled water. The capsules were dried in a laminar airflow and then dissected longitudinally with a surgical blade. The seeds were sown by spreading as thinly as possible over the surface of culture medium in 22×120 mm glass test tubes (approximately 10 test tubes per capsule), each containing 10 ml of medium. The medium consisted of half-strength Murashige and Skoog’s (MS) basal medium with 3% sucrose, solidified with 0.9% Difco agar. Three capsules (thirty tubes) were evaluated per species, and the cultures were incubated for 16 weeks. The percentage of green healthy seedlings, and green evaluated per species, and the cultures were incubated for 16 weeks of culture at 25 °C day/night temperature until flowering. A voucher specimen of each plant species has been deposited at the China Medical College herbarium, Taichung, Taiwan. *Dendrobium tosaense* MAKINO CMC DT 0303; *Dendrobium monoliforme* Sw CMC DM 0302; and *Dendrobium linawanum* REICH. f. CMC DL.0301.

**Transfer of Plants to Greenhouse for Hardening** The plants from each species, with well developed rhizomes and shoots, were washed thoroughly under tap water for 2—3 min to remove traces of agar-gelled medium sticking to them. These were then planted in plastic trays (diameter 6 cm×height 5.5 cm×20 hole) containing moss and tree fern (1 : 1 vol : vol) or moss as a substrate and kept in the greenhouse. The plants were initially covered with a polythene sheet for one month to maintain high humidity (above 70%), and were irrigated twice a week with tap water. After one month the plants were irrigated once in a week.

**1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity** Tissue culture raised plants grown for six months in a greenhouse were collected and freeze-dried in a lyophilizer (FTS System, New York, U.S.A.). The dried plants (5 g) were finely ground with mortar and pestle and extracted independently with methanol (50 ml×3) under 20 min sonication (Branson Ultrasonic Cleaner, Branson Cleaning Equipment Co., Shelton, CT, U.S.A.) to ensure the complete extraction. These extracts were filtered through an Advantec No.1 filter paper (Toyo Roshi Kaisha Ltd., Japan), and the methanol was evaporated in vacuo to dryness. The dry residue was further extracted with methanol and water. The methanol solubles were dried over anhydrous sodium sulphate and concentrated to get the crude residue under reduced pressure.

Scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals of *Dendrobium* extracts was measured according to the method reported by Blois31 with minor modifications. Each sample stock solution was diluted to final concentrations of 400, 200, 100, 50 and 25 μg/ml. 0.2 ml of methanol and 0.3 ml of various concentrations of the samples in methanol was mixed in a 10 ml test tube. To this, 2.5 ml of 75 μM DPPH in methanol was added to achieve a final volume of 3 ml. The solution was kept at room temperature for 90 min and the absorbance at 517 nm (ε417) was measured. α-Tocopherol was used as reference compound. The DPPH scavenging effect was calculated as follows:

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\text{scavenging effect} \% = \frac{(A_0 - (A - A_b))}{A_0} \times 100
\]

Where: \(A_0\): \(A_{417}\) of DPPH without sample; \(A\): \(A_{417}\) of sample and DPPH; \(A_b\): \(A_{515}\) of sample without DPPH.

**Statistical Analysis** The tissue culture data was analyzed using the least significant difference test. The DPPH Radical Scavenging activity of the methanolic extracts is expressed as the concentration required to effect 50% inhibition of decreasing signal peak height (IC50), and the samples were analyzed two times.

**RESULTS AND DISCUSSION**

**Plant Materials and Pollination** Healthy growth and a uniform flowering pattern was observed in the plants growing under controlled greenhouse conditions. For carrying out artificial cross-pollination, it is necessary to obtain simulta-
neous flowering in different plants. A synchronized flowering pattern has been observed in plants grown in a greenhouse. Also, explants taken from the plants growing in controlled environmental conditions could be easily established in tissue culture.

**Seed Culture and Germination** Data on the response of seeds obtained from capsules after 12 weeks of hand pollination for the two species *D. moniliforme* and *D. tosaense* after 16 weeks of culture on half-strength MS basal medium has been tabulated in Table 1. Seed germination varied with the *Dendrobium* species. Seventy-four percent of the seeds developed into healthy seedlings, and only 1.5% of white PLB were observed when cultured for 16 weeks on half strength MS basal medium (Fig. 1A). Tissue culture has been successfully employed for the propagation of orchid species. Germinating orchid seeds asymbiotically in sterile culture conditions is one of the methods of conservation and propagation of orchids. Immature capsules have been used for the propagation of orchids, as they could be easily surface sterilized without causing more damage to seed viability.

Plant growth regulator, especially cytokinin, plays an important role in orchid seed germination, however, in the present study the *Dendrobium* seeds germinated on medium devoid of cytokinin. This may be because of the presence of a sufficient level of endogenous cytokinin required for the initial stages of germination.

**Growth of Seedling** The sub-culture of seedlings on the fresh medium of similar combination was not beneficial for further growth. Therefore, seedlings were subcultured on medium supplemented with 8% banana homogenate, 8% potato homogenate and 8% coconut milk to achieve profuse growth. A significant variation in the growth pattern was observed in the four-month-old seedlings obtained from the seeds derived from 12-week-old capsules cultured on the basal medium with various addendums (Table 2, Figs. 1B—D). In orchids, organic additives such as coconut milk and/or banana homogenate and/or potato homogenate, added to the medium on seedling growth, supported the healthy growth of the shoots and/or seedlings. Healthy seedlings of the three species of *Dendrobium* were transferred on moss or...
moss and tree fern for *ex vitro* establishment of plants. 

**Transfer of Plants to Greenhouse** The plants were successfully hardened in the greenhouse (Fig. 1E). A high acclimatization rate (84—100%) of plants was obtained by maintaining high humidity (70%) conditions in the greenhouse under natural light and 25/20 °C day/night temperature (Table 3). The high humidity conditions prevented the withering of healthy, nine-month-old seed-derived plants transferred to moss or moss and tree fern substrates. The tissue culture raised seedling derived plants were morphologically similar to the naturally grown plants. Plants grown in the greenhouse were harvested and used to study the DPPH activity.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity Currently there is much interest in the production of low-density lipoprotein in important cells and organs, as well as in food systems, to act against oxidative damage caused by superoxide, hydroxyl and peroxyl radicals. Obviously, there has been an increasing demand to evaluate the antioxidant properties of direct plant extracts or isolated compounds of plant origin rather than synthetic ones.  

Since the DPPH system is a stable radical generating procedure and can accommodate a large number of samples in a short period, and is sensitive enough to detect active principles at lower concentrations, it was used in the present study for primary screening of the antiradical activities of the methanolic extracts of *D. tosane* MAKINO, *D. monoliforme* Sw., and *D. linawanum* REICHB. f. by UV spectrophotometric assay. DPPH is a stable free radical that shows maximum absorption at 517 nm in methanol. When DPPH encounters a proton donating substance, for example, an antioxidant, the radical would be scavenged and the absorbance at 517 nm is reduced. Based on this principle, the antioxidant activity of a substance can be expressed as its ability to scavenge the DPPH free radical. The methanolic extracts of *Dendrobium* species are known to contain a wide variety of potentially useful chemical compounds that include alkaloids, fluorenones and sesquiterpenoids, stilbenoids, alkyl ferulates. Some of these chemical constituents, like stilbenoids, alkyl ferulates, aromatic phenolic compounds are known to possess antioxidant activity. The antioxidant activity of methanolic extracts obtained from three *in vitro* propagated species of *Dendrobium* has been studied. Plants propagated by *in vitro* techniques showed less variation in the content of secondary metabolites than their cultivated/wild counterparts. Also, *in vitro* propagated plants are found to contain higher amounts of active ingredients than the intact plants. The plants belonging to the genus *Dendrobium* were chosen to be evaluated for their antiradical capacity due to the presence of secondary metabolites that exhibit some interesting pharmacological properties. The mean inhibition rate of 50% (IC$_{50}$ values) was calculated (Table 4). All the extracts scavenged the DPPH radical significantly in a concentration dependent manner. The order of the antioxidant activity was: *D. tosane* > *D. linawanum* > *D. monoliforme*. The dosage of extract is expressed in milligrams of dry weight of the extract per milliliter of the assay mixture. The results should be further examined in future *in vivo* studies, which could ultimately lead to the application of these medicinal plants in pharmaceutical preparations.

**CONCLUSIONS**

A simple protocol for asymbiotic seed germination of three species of *Dendrobium* has been described. The free radical scavenging activity of total crude extracts of three species of the genus *Dendrobium* have been quantitatively determined using DPPH assay in this present investigation. The results obtained from the present studies may give a rational explanation for the therapeutic use of the herbs of the genus *Dendrobium* in Chinese medicine. However, characterization of the active principles of these plants with radical scavenging action is needed and is in progress in our laboratory.

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