ABSTRACT

Eulophia nuda Lindl [Synonym: *E. spectabilis* (Dennst.) Suresh] is a highly medicinal terrestrial orchid grows up to 75 cm with underground tubers and dark pinkish colour flowers (April-July), found in Southeast Asia. In India, this plant is found in tropical Himalayas (Uttarakhand, Assam, Arunachal Pradesh) and Western Ghat (Maharashtra, Karnataka, Kerala). Ethanobotanical studies revealed that its tubers are used against tumors, scrofulous glands of the neck, bronchitis, blood diseases, skin rash, rheumatoid arthritis, acidity, piles and stomach complaints, snake bite, tuberculosis, besides being used as a vermifuge, appetizer and an aphrodisiac drug. Further, pharmacological activities including anti-proliferative activities against cancerous cells, DNA damage protecting activities, anti-glycation, anti-inflammatory, antibacterial, antifungal and hepatoprotective activities have been reported from this plant. Though *E. nuda* is being used against many diseases by tribal communities and local healers, there are very few reports on scientific validation of its medicinal properties. Owing to its high therapeutic values, natural populations of *E. nuda* are under threat from over-exploitation and figured prominently in the Red Data book of IUCN and therefore need immediate steps for its conservation and large-scale propagation.

Considering the importance of this plant and urgent need to conserve this plant, *in-vitro* tissue culture protocol was developed for *E. nuda* Lindl in which asymbiotic seed germination as well as direct and indirect organogenesis were successfully done. *In-vitro* asymbiotic seed germination is very crucial as orchids usually lack endosperm, and natural germination rate is very low and they often require fungal associations for their germination. In the present study, the seeds of different ages were germinated on half and full-strength MS (Murashige and Skoog), KC (Knudson) and BM1 terrestrial orchid (Van Waes and Debergh) media. Amongst various supplements, 15% coconut water (CW) and 500 mg l^{-1} casein hydrolysate proved beneficial for seed germination. BM1 fortified with CW showed the highest germination (80.5 ± 2.1%) after 60 days of inoculation, which was further improved to 91.3 ± 1.9% upon incorporation of 1 mg l^{-1} each of α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) and eventually more than 50% cultures developed into seedlings. BM1 containing CW nourished with 2.5 mg l^{-1} BAP and 1.5 mg l^{-1} kinetin (Kin) proved best for multiple shoot induction from seedlings. Shoot numbers increased further with successive transfers onto the same medium. Sixty shoots per shoot clump were achieved after four cycles of 30 days each. Microshoots were rooted on MS

containing 2.0 mg l^{-1} indole-3 butyric acid (IBA) and 200 mg l^{-1} activated charcoal with 81.3 ± 2.4% efficiency. Rooted plantlets were gradually acclimatized to greenhouse (70% survival) and they exhibited normal morphology and growth characteristics. Flow-cytometry based DNA content analysis revealed that the ploidy levels were maintained in *in-vitro* propagated plants.

For direct and indirect organogenesis, various parameters including explant-type, medium compositions, use of phytohormones and additives (adenine sulfate, arginine, ascorbic acid and citric acid) were optimized for direct and indirect regeneration of *E. nuda*. Protocorms proved the best explants for shoot initiation, proliferation and callus induction. The MS medium containing 2.5 mg l^{-1} 6-benzylaminopurine (BAP), 1.0 mg l^{-1} Kin and additives (adenine sulfate, arginine, citric acid, 30 mg l^{-1} each and 50 mg l^{-1} ascorbic acid) was found optimal for shoot multiplication (12.1 shoots and 7.1 PLBs per explant with synchronized growth), besides producing basal-callus. Shoot number was further increased with three successive subcultures on the same media and ~40 shoots per explant were achieved after 3 cycles of 30 days each. Additives (ascorbic acid, adenine sulfate and citric acid) and casein hydrolysate (CH) showed advantageous effects on indirect shoot regeneration via protocorm-derived callus. Optimum indirect regeneration was achieved on MS containing additives, 500 mg l^{-1} CH, 2.5 mg l^{-1} BAP and 1.0 mg l^{-1} Kin with 30 PLBs and 6 shoots per callus mass (~5 mm size). The shoots were rooted (70% frequency) on ¼-strength MS medium containing 2.0 mg l^{-1} IBA, 200 mg l^{-1} activated charcoal and additives. The rooted plantlets were hardened and transferred to greenhouse with 63% survival rate. Flow-cytometry based DNA content analysis revealed that the ploidy levels were maintained in *in vitro* regenerated plants.

In-vitro models confirmed strong dose-dependent antioxidant potentials of *E. nuda* tuber extracts. Petroleum ether (PEE), ethyl acetate (EAE), methanol (ME) and aqueous methanol (AqME) extracts (200-1000 µg ml^{-1} each) also showed significant free-radical scavenging potencies. Overall, the antioxidant efficacies were in the order EAE>ME>AqME>PEE. *In-vitro* cytotoxicity of two most-active extracts, EAE and ME were evaluated against human cancerous cells (MCF-7). EAE showed striking cytotoxicity, as only 0.86% cell survived at 1000 µg ml^{-1} concentration, whereas 5.17% survival was recorded in presence of ME at same concentration. To identify and characterize phytochemical constituents, LC-ESI/MS profiling of most potent extract EAE was carried out, which revealed 37 identified compounds including catechin,

taxifolin, tocopherol, trigallic acid, pinoresinol and chlorogenic acid, all known for their strong antioxidant/anticancer properties.

Phytochemical constituents and antioxidant efficacies of solvent-based extracts of *E. nuda* were investigated. Besides, the molecular mechanisms underlying strong antioxidant and antiproliferative activities, especially the via up-regulation of nuclear transcription factor-erythroid 2 related factor (*Nrf2*) and hemeoxygenase-1 (*HO-1*) pathways was assessed, as *Nrf2* is known as master regulator of antioxidant enzymes and *HO-1* is its one of the target. *Nrf2*/*HO-1* pathway has turn into a striking target for prevention and treatment of oxidative stress-associated maladies like cancer, neurodegenerative, cardiovascular, and inflammatory diseases. The expression levels of two key-factors in antioxidant systems- *Nrf2* and *HO-1* were therefore analyzed by qRT PCR in EAE-treated MCF-7 cells. Expression levels of both genes were upregulated beyond 50 µg ml^{-1} extract concentration with > 2 - fold increase at 200 µg ml^{-1} EAE. Collectively, the data demonstrated that *E. nuda* extracts possess strong free radical scavenging and antioxidant efficacies and the mechanism of action be via inducing *Nrf2* and *HO-1*.

It was observed that phenolics were highly correlated with antioxidant activity and HR-LC-MS proofing revealed presence of phenolic compounds including gallic acid, catechin, vanillin and quercetin etc and were mainly responsible for antioxidant activity. Hence the phenolics were elicited in *in-vitro* cultures using biotic and/or abiotic elicitors. Two endophytic fungi, fungal isolate III and fungal isolate IV identified as *Fusarium proliferatum* (Matsush.) and *Talaromyces pinophilus* (Hedgc.) on the basis of micromorphological and molecular characteristics (ITS4 and ITS5) with accession numbers MH636868 and MH685524, respectively. Water extracted mycelial polysaccharide from endophytic fungi isolated from rhizomes were used as biotic elicitors while methyl jasmonate (MJ) as signaling molecule, phenyl alanine (PA), sodium chloride (NaCl) and Calcium chloride ($CaCl_2$) were used as abiotic elicitors. The biomass was harvested after two months. The growth was measured on the basis of fresh and dry weight of harvested biomass. The samples were dried in oven at 40ºC till they attained a constant weight. The dried samples were further employed for estimation of total polyphenols. Among the all biotic and abiotic elicitors; MJ and WEMP from F-IV (*Talaromyces pinophilus*) elicited the polyphenol in both callus as well as shoot culture when used singly.

Synergistic effects of biotic and abiotic elicitors were observed when cultures were treated with 100 mg l^{-1} WEMP F-IV (*Talaromyces pinophilus*) + 100µM MJ, as polyphenol production increased in shoot cultures. Estimation of individual phenolics including gallic acid, catechin, vanillin and quercetin showed their elicited production in shoot cultures with a clear indication of synergistic effect of WEMP and MJ, which was further confirmed by HPLC based estimation. Overall, 3.01-, 2.01-, 1.79-, 1.65-fold increase in gallic acid, catechin, vanillin and quercetin content, respectively, was recorded in *in-vitro* shoots over wild tubers (*in vivo*) , whereas 7.54-, 3.13-, 2.03-, 1.73-fold increase, respectively, was evidenced in treated shoots apparently as a synergistic effect of biotic and abiotic elicitors [100 mg l^{-1} F-IV (*Talaromyces pinophilus*) + 100µM MJ].

Asymbiotic seed germination, direct and indirect organogenesis of *E. nuda*, antioxidant and anti-proliferative potentials of solvent extracts of this orchid along with deciphering the molecular mechanisms underlying these strong activities via studying the *Nrf2/HO-1* gene expression patterns in human cells treated with potent extract. The elicitation of important phenolics in *in*-vitro cultures of *E. nuda* using biotic and abiotic elicitors was also achieved successfully.