ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 05, pp.16383-16386, May, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

EFFECT OF CYTOKININS ON SHOOT INDUCTION FROM SEED DERIVED RHIZOMES IN *EULOPHIA NUDA* LINDL

***Varsha Dawande and Rajaram Gurav**

Department of Botany, Shivaji University, Kolhapur, India

ARTICLE INFO

Article History:
Received 05th February, 2015
Received in revised form
23rd March, 2015
Accepted 17th April, 2015
Published online 31st May, 2015

Key words:
BA,
Kn,
TDZ,
Eulophia nuda.

ABSTRACT

Eulophia nuda, a terrestrial, endangered orchid was clonally propagated using seed derived *in-vitro* grown rhizomes. Cytokinins are most commonly used plant growth regulators, to stimulate cell division and morphogenesis in tissue culture. The effect of commonly used cytokinins, N^6 Benzylaminopurine, Kinetin and Thidiazurone was studied for induction of shoots from *in vitro* grown rhizomes. The rhizomes were cultured and proliferated on Murashige and Skoog's medium fortified with cytokinins with different concentrations. Among the three cytokinins tested BA (6.66µM) showed best results with respect to number of shoots induced and length of shoots. (4.51±1.6 number of shoots per explant and ~ 5cm length) within 20 days. Kn was found to be least effective for shoot induction. TDZ induced shoots were less in number and also stunted as compared with BA. The regenerated shoots easily developed roots in various strengths of MS medium (75% in 1/4 MS with 5 roots/shoot and 2cm length). The regenerated plantlets were successfully acclimatized and transferred to earthen pots.

***Citation*:** Varsha Dawande and Rajaram Gurav, "Effect of Cytokinins on shoot induction from seed derived Rhizomes in *Eulophia nuda* Lindl", *International Journal of Current Research*, 7, (5), 16383-16386.

INTRODUCTION

Orchidaceae is one of the most diverse plant families, with more than 800 described genera and about 25,000 species. They are prized for their beautiful flowers having an incredible range of size, shape and colour. Many orchid species are also used as medicine by alternative systems of treatment like Ayurveda and traditional Chinese medicine. Some orchids are facing an uncertain future through unscrupulous collection, the impacts of climate change and habitat loss, complex requirements for pollination, constraint in seed germination and many other biotic and abiotic factors contributing to loss of plants in wild when these requirements are not met (Reed *et al.,* 2011).

The genus *Eulophia* is a perennial, terrestrial orchid found in Central and Southeast Asia region with 22 species from India. *Eulophia nuda* Lindl. [Synonym: *Eulophia spectabilis* (Dennst.) Suresh] is medicinally important, used in treatment for tumors, various health problems by the local healers throughout the Western Ghats region in Maharashtra (India). In Thailand, this orchid is used for treatment of skin rash.

****Corresponding author: Varsha Dawande,***
Department of Botany, Shivaji University, Kolhapur, India.

Raw rhizomes are eaten for curing rheumatoid arthritis (Mali and Bhadane, 2008). It is reported to be demulcent and antihelmintic in action (Singh and Duggal, 2009). The rhizomes are used to treat acidity, piles and stomach complaints (Mahekar and Yadav, 2006) and also as aphrodisiac drug (Jagdale *et al*., 2009). Nine different phenanthrenes, a rather uncommon class of aromatic metabolites, have been reported from *E. nuda* till date (Kovacs, 2008). An amorphous phenanthrene, named nudol, later identified as 2,7-hydroxy-3,4-dimethoxyphenanthrene has been isolated from Eulophia nuda, E. ohreata**,** Erica carinata and Erica stricta (Merchant *et al*., 1962; Bhandari *et al*., 1985; Datla *et al*., 2010; Kshirsagar *et al*., 2010). Phenanthrene derivative 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol, isolated from rhizomes showed promising cytotoxic activity against human cancer cells (Shrirama, 2010).

Eulophia nuda is mainly propagated through the rhizomes in nature and they produce only limited number of propagules. It is too slow process to propagate large quantity clones as the seeds lack endosperms and require mycorhizal association for germination. Further, the outbreeding tendency hinders propagation of elite genotypes through seeds. This plant is under sever threat due to unscientific management practices, ever increasing demand for phytochemicals and destruction of

its natural habitat. Therefore, it is important to establish an efficient regeneration and multiplication system for the production of large number of rhizomatous plantlets.

Tissue culture techniques for micropropagation of orchids are well known. Also the growth regulators play an important role for subsequent development of protocorms. Cytokinins are effective for induction and differentiation of shoot buds. In present study three different cytokinins were tested for their efficiency in induction of shoot buds from seed derived rhizomes.

MATERIALS AND METHODS

Plant material, surface sterilization and explant preparation

Capsules of *Eulophia nuda* maintained in Botanical garden, Shivaji University, Kolhapur, India were harvested in the month of October before dehiscence. The capsules were washed with liquid soap under tap water and further surface sterilized by dipping in 70% ethanol for 30 seconds in Laminar air flow unit.

Fig. 1.
1, 2 and 3: Asymbiotic seed germination and development of seedlings
4: Rhizome from 4-5 month old seedling
5: Multiple shoots developed from rhizome explants
6: Individual plantlet
7: Acclimatized plants

Culture medium and culture conditions

The capsules were cut open and seeds sowed aseptically in petri dishes (Laxbro India 88X55mm), containing Knudson media (Knudson 1946) fortified with 3% (w/v) sucrose and 0.9% agar (Himedia) as gelling agent without addition of plant growth regulators. The small protocorms obtained were transplanted on to Murashige and Skoogs (1962) medium supplemented with 2% (w/v) sucrose and 0.8 % agar (himedia) as gelling agent. The rhizomes derived from aseptically grown seedlings were cut transversely into pieces ~ 1cm^2 and inoculated on MS medium containing various concentrations of N^6 Benzylaminopurine (2.22-8.88 µM), Kinetin (0.92-4.61 µM) and Thidiazurone (0.90-9.08 µM). Subsequent transfer to fresh medium was carried out according to growth rate, usually after every 20 days. The cultures were observed constantly for any response and results recorded after every 20 days. Well developed shoots ~ 5-6 cm length were transferred to MS medium of 1/2, 3/4, 1/4 and full strength devoid of plant growth regulators for rooting. The pH of the medium was adjusted to 5.8±0.02 using 1 N NaOH or 0.1N HCL prior to autoclaving for 15 min at 15psi and 121 ^{0}C . The cultures were maintained at 28± 2^{0}C under 12 h d^{-1} photoperiod with a white cool fluorescent light (40µMolm^{2}s^{-1}).

Acclimatization of regenerated plantlets

Rooted plantlets with fully expanded leaves and well developed roots were transferred to pots containing sand and soil (1:1) and maintained in controlled growth chamber conditions at 25 ± 1ºC, 16 hrs photoperiod and 80% relative humidity followed by gradual shift to green house conditions.

Experimental design and statistical analysis

All the experiments were conducted with the minimum of 30 replicates per treatment and the experiments were repeated three times. MS medium without growth regulator is used as control in all experiments. The results are expressed as mean ± SD of three experiments. Data were subjected to analysis of variance (ANOVA) and means were compared by Duncan's multiple range test at $P<0.05$ using the SPSS ver.21 (IBM SPSS ver 21).

RESULTS AND DISCUSSION

Seed germination and seedling development

Asymbiotic seed germination started relatively slowly within 8-9 weeks of culture where embryos swelled and broke out of the testa, and then formed green protocorms after 11-12 weeks of sowing on Knudson C medium (1946) without addition of plant growth regulators. Subsequent transfer to fresh Murashige and Skoog's (1962) medium was carried out according to growth rate and stage of development, usually after each 4 to 6 weeks for seedling development. The branched mini rhizomes differentiated from protocorms produced shoot and roots within 6-8 weeks. About 75 % of seeds germinated and developed seedlings. The rhizomes from 4-5 months old and vigorous seedling were used as explants to study effect of cytokinins on multiple shoot induction.

Effect of BA on shoot induction

The effect of BA with different concentrations on shoot induction is shown in Table 1. BA is most potent cytokinin for inducing direct shoot formation as reported in *Cymbidium*

forrestii (Paek and Yung 1991) and *Geodorum densiflorum* (Sheelavantmath *et al*., 2000). In present study number of shoots induced increased with concentration of BA up to 6.66 µM which show best results for multiple shoot induction in 100 % explants with 4.51±1.6 number of shoots per explant and ~ 5cm length in 20 days. The number further increased upto 8 shoots during 3rd subculture. However further increase in concentration (8.88 µM) reduced the number and length of shoots.

Table 1. Effect of BA on shoot induction in rhizomes of *Eulophia nuda*

BA (µM)	% response	No.of shoots(cm)	Length of shoots (mean cm)
control	30	2.13±0.61c	1.42±0.88e
2.22	34	2.73±0.78c	1.60±0.30d
4.44	85	2.96±1.06b	2.68±0.26c
6.66	100	4.53±1.60a	4.49±0.20a
8.88	90	3.16±1.04b	3.39±0.39b

Basal medium: MS supplemented with 2% (w/v) sucrose and 0.8% (w/v) Agar. Means followed by the different letter within column are significantly different (P<0.05) using Duncan`s multiple range test.

Effect of Kn on shoot induction

The effect of Kn with different concentrations on shoot induction is shown in Table 2. Kinetin has been shown to have a morphogenetic response in processes associated to micropropagation of orchid species (Martin, 2003). However, in some cases, it is a growth inhibitor of callus tissue (Tokuhara and Mii, 2003). Cytokinins are good inducer of protocorm-like bodies from leaf explants (Murthy and Pyati, 2001; Lee and Lee, 2003). In our experiment multiple shoot induction was found to be low in medium supplemented with Kn. Maximum 66% response was observed in medium supplemented with 4.64 µM Kn, but the number of shoots were less as compared to lower concentrations of Kn.

Table 2. Effect of Kn on shoot induction in rhizomes of *Eulophia nuda*

Kn (µM)	% response	No.of shoots(cm)	Length of shoots (mean cm)
Control	30	2.13±0.61b	1.42±0.88bc
0.92	30	1.76±0.44c	1.52±0.08b
1.85	40	2.36±0.56a	1.05±0.14d
2.32	45	2.46±0.50a	1.39±0.82c
4.64	66	1.63±0.55c	1.88±0.23a

Basal medium: MS supplemented with 2% (w/v) sucrose and 0.8% (w/v) Agar. Means followed by the different letter within column are significantly different (P<0.05) using Duncan`s multiple range test

Effect of TDZ on shoot induction

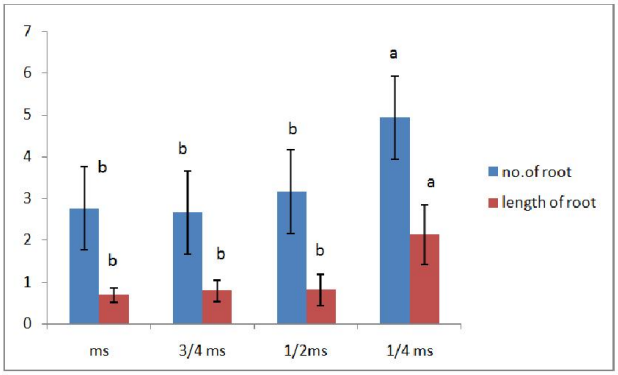
The effect of TDZ with different concentrations on shoot induction is shown in Table 3. TDZ is a substituted phenyl urea with cytokinin-like activity, useful for plant regeneration from several species through organogenesis and promotes *in vitro* shoot formation in several orchid species. TDZ is effective in inducing *in vitro* morphogenesis especially shoot regeneration and proliferation (Ernst, 1994; Chen and Piluek, 1995; Nayak *et al*., 1997 and Chen and Chang, 2000) and direct somatic embryogenesis (Chen *et al*., 1999; Chen and Chang, 2000a). In present study TDZ induces multiple shoots in about 77% explants. Maximum numbers of shoots were observed in medium with 9.08 µM of TDZ. The shoot number increased with concentration of TDZ (0.90-4.54µM) but not significantly from control.

Table 3. Effect of TDZ on shoot induction in rhizomes of *Eulophia nuda*

TDZ (µM)	% response	No.ofshoots(cm)	Length of shoots (mean cm)
Control	30	2.13±0.61c	1.42±0.88d
0.90	37	2.23±0.49c	2.00±0.20c
2.27	40	1.96±0.81c	2.89±0.32b
4.54	77	2.90±0.65b	4.22±0.29a
9.08	66	3.93±1.16a	2.02±0.33c

Basal medium: MS supplemented with 2% (w/v) sucrose and 0.8% (w/v) Agar. Means followed by the different letter within column are significantly different (P<0.05) using Duncan`s multiple range test

Well developed shoots of ~ 5-6 cm length were transferred to MS medium of 1/2, 3/4,1/4 and full strength devoid of plant growth regulators for rooting. Almost 75% shoots rooted *in-vitro*. In ¼ MS medium the number and length of root was more than other medium strength used (Graph 1).



Graph 1. Effect of various strength of MS medium on root induction

Basal medium: different strength of MS supplemented with 2% (w/v) sucrose and 0.8% (w/v) Agar. Means followed by the different letter are significantly different (P<0.05) using Duncan`s multiple range test

Multiplication of orchid species is possible using different types of explant. Protocorms were effective in a few *Eulophia* species (McAlister and Van Staden 1998); rhizome segments from adult plants in *Eulophia nuda*, *Geodorum purpureum,* and *Satyrium nepalense* (Panwar *et al.,* 2012; Mohapatra and Rout, 2005, Mahendran and Bai, 2009) and segments of mini-rhizomes differentiated from protocorms were successful for *G. densiflorum* (Sheelavantmath *et al*., 2000). Rhizomes developed in seedlings that had nodes and internodes were equivalent to whole rhizomes from adult plants, which was also a good explant source as proven in the present study Cytokinins are used in tissue culture to stimulate cell division and control morphogenesis. The three cytokinins tested, all promoted direct shoot formation in rhizome sections. The concentration of cytokinins has been reported to be decisive for shoot proliferation. The shoot buds were developed from explants within 20 days of inoculation which further grows up to 4.5 cm in 4-5 weeks. The 100% response was observed in medium supplemented with 6.66µM BA. Shoot bud induction was found least in medium supplemented with different concentrations of Kn. The number and shoot length produced in medium containing Kn was not much different than control.

Similar results were observed in *Dendrobium nobile* (Hafiz *et al.,* 2011), where minimum number of shoots were produced in medium fortified with Kn. It was seen that TDZ is more effective than other cytokinins in inducing shoot bud differentiation from various explants (Earnst, 1994; Nayak *et al.,* 1997). In present study effect of TDZ on induction of shoot buds was found to be less than BA with respect to number and length of shoots. Further, elongation of shoots was also reported to be slower and less as compared with BA. Overall, BA appeared to elicit the best shoot multiplication in response compared with either Kn or TDZ. In present study rhizomes from *in vitro* grown plantlets were used as a source of explants, having advantages as, availability of explants without damaging the natural source and minimum contamination since they are obtained from aseptically grown plants. Further the concentration of BA is much less as compared with that of previously reported (Panwar *et al*., 2012), minimizing the chances of genome instability or variation in ploidy level due to high levels of exogenous PGR in culture medium. To conclude, the present study describes an efficient and relatively easier method for regeneration and micropropagation of *Eulophia nuda*. Multiple shoots were induced from *in vitro* raised rhizome explants with minimum PGR in relatively shorter period, which successfully rooted and survived. The results may hold a key for mass multiplication and conservation of this endangered terrestrial orchid with therapeutic potential.

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