Biosciences, Biotech. Research Asia Volume 02, No.(1) 65-70 (2004)

NUCLEIC ACID AND SOLUBLE PROTEIN CONTENTS AT DIFFERENT STAGES OF PROTOCORM DEVELOPMENT OF *DENDROBIUM FIMBRIATUM* VAR. *OCULATUM* HK. F. AS INFLUENCED BY GROWTH REGULATORS

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(Received, May 05, 2004)

ABSTRACT

The effects of indole-3-acetic acid, indole-3-butyric acid, α - naphthalene acetic acid, 2, 4-dichlorophenoxyacetic acid, 6- furfurylaminopurine (kinetin) and 6- benzylaminopurjne were studied on the nucleic acids (DNA and RNA) and soluble protein contents at different stages of protocorm development of *Dendrobium fimbriatum var. oculatum* Hk. f. Compared to the untreated controls, DNA and RNA contents were higher in most of the treated protocorms. However, in case of 2, 4-dichlorophenoxyacetic acid treatment, the nucleic acid contents decreased after the emergence of leaf initials. The growth regulators also markedly affected the synthesis of soluble proteins. The soluble protein content increased with growth except in protocorms treated with higher concentrations of 2, 4- dichlorophenoxyacetic and 6- benzylaminopurine.

Key words: DNA, RNA, Soluble protein, Protocorm development.

INTRODUCTION

Orchid seeds germinate in nature symbiotically following fungal infection[1,2]. Asymbiotic seed germination can be achieved on suitable medium supplemented with growth regulators. Reports show that the protocorms in asymbiotic cultures enlarge and differentiate at a slower rate as compared to the ones having symbiotic association. The extremely slow growth of the orchid *Taeniophyllum aphyllum* and the much faster rate of species like *Zeuxine strateumatica* could be due to the differences in the rates of DNA synthesis[3]. DNA content of the parenchyma cells of *Vanda* ovules was reported to increase together with that of RNA and nuclear size[4]. The differences in the ability of seedlings to synthesize DNA in vitro with or without mycorrhizal infection accounts for the differences in the germination capability of the orchid seeds. Differentiation of the *Cymbidium* protocorms as a result of the effect of plant growth regulators on the DNA content of the cells was reported earlier by Nagl and Rucker[5]. No further work on this aspect was done in orchids and it is not clear whether the report of Nagl and Rucker[5] holds true for the other orchid species as well. However, it is generally believed that morphogenesis and differentiation in many plants is associated with the nucleic acids and proteins, the synthesis of which is regulated by growth regulators[6,7,8]. The present communication is aimed at studying the effect of growth regulators on the nucleic acid and protein contents in axenic protocorms of an economically and commercially important orchid, *Dendrobium fimbriatum* var. *oculatum*, at different stages of development so as to understand the phenomenon of growth in them.

MATERIAL AND METHODS

a) Experimental material:

Seeds of *Dendrobium fimbriatum* var. *oculatum* Hk.f. were germinated on Murashige and Skoog, MS[9] medium devoid of growth regulators[10]. One-month old developing green protocorms were transferred to fresh MS medium supplemented with different growth regulators (indole-3-acetic acid, IAA; a-naphthaleneacetic acid, NAA; indole-3-butyric acid, IBA; 2,4-dicholorophenoxyacetic acid, 2,4-D; 6-benzylamino purine, BAP; kinetin, KN; Gibberellic acid, GA_3) incorporated at concentrations ranging from 0.0-10.0 mM. The control comprised protocorms grown in MS medium devoid of growth regulators. The cultures were incubated at 25±2 °C temperature and 16 h photoperiod of light intensity 150 mmoles $m^{-2}sec^{-1}$ provided by fluorescent lamps. Five replicates of each of the treatments were taken and the experiments were repeated twice.

The estimations of soluble proteins and nucleic acids (DNA and RNA) were carried out in the developing protocorms and four different stages of protocorm were recognized:

Stage I : Protocorms at 15 days of culture
Stage II : Protocorms showing pointed vegetative apex at 30 days of culture
Stage III : Enlarged protocorms showing leaf initials at 45 days of culture
Stage IV : Protocorms at two-leaf stage showing root initials at 60 days of culture

b). Soluble protein estimation:

The method of Lowry *et al.*[11] was followed for the estimation of soluble proteins. 500 mg each of the treated tissue was extracted in alcohol and macerated in chilled mortar with pestle. The volume was made up to 5 ml with 80 % alcohol and centrifuged at 5000 rpm for 20 min. The supernatant was discarded and the residue suspended in 5 % trichloroacetic acid (TCA) for 15 min after which 2 ml of its aliquot was taken and resuspended in 10 % TCA in the ratio of 1: 1 (v/v) for 15 min to precipitate proteins at 0-2 °C, and centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the residue washed twice with distilled water, dissolved in 1.0 ml of 0.5 N NaOH, and diluted to desired volume. Suitable volume of this solution was taken and 5.0 ml freshly prepared alkaline solution (1.0 ml of 0.3 % copper sulphate in 1 % sodium-potassium tartarate mixed with 50 ml of 2.0 % sodium carbonate solution) was added and allowed to stand for 10 min at room temperature. After incubation, 0.5 ml of Folin-Phenol reagent (equally diluted with water) was added with immediate shaking and held at room temperature for 30 min and the colour was read at 750 nm. Soluble protein was expressed as mg per 100 mg fresh weight tissue using standard curve prepared by bovine serum albumin. The tissue extract was subjected to PAGE according to Ornstein[12] and Davis[13] and the gels stained for soluble proteins using 0.1% amido black in 7 % acetic acid.

c). Nucleic acid estimation:

DNA and RNA contents of the tissues were estimated by means of diphenylamine and orcinol reactions respectively, as described by Plummer[14]. TCA precipitates of the plant tissues (obtained during the preparation of samples for protein estimation) were used for the estimation of DNA and RNA. The residue was suspended in suitable volume of 0.3 N KOH and incubated for 16-18 h at 37 °C. It was then centrifuged at 5000 rpm for 15-20 min and the supernatant was collected. The residue was washed twice with distilled water, centrifuged and the supernatant collected each time. The supernatants were pooled together and the final volume made up to 15 ml with distilled water. This extract contained the nucleic acids. For the separation of DNA and RNA, the nucleic acid extract was acidified to pH 1.5 with perchloric acid. This precipitated DNA and excess of potassium chlorate. The extract was centrifuged at 5000 rpm for 15 – 20 min and supernatant containing RNA and residue containing DNA were collected.

The RNA present in the supernatant was estimated by orcinol reaction whereas DNA was estimated by diphenylamine reaction after solubilizing the residue in perchloric acid and removing the excess of perchlorate.

i). Estimation of RNA

An appropriate amount of the RNA extract was mixed with 3.0 ml of orcinol reagent (1.0 g of ferric chloride dissolved in 1 litre of conc. HCl and to it was added 35 ml of 6 % w/v orcinol in alcohol). It was then heated in a boiling water bath for 20 min, cooled and the absorbance read at 665 nm against the orcinol blank. The total RNA content was expressed in mg per 100 mg fresh weight.

Clarification of DNA

The residue containing DNA was suspended in suitable aliquots of 5 % of TCA at 0 °C and centrifuged at 5000 rpm for 15 min. The supernatant was discarded and the process was repeated twice. The residue was washed with absolute ethanol and then with ethanol : ether (3 : 1) mixture. The residue was then suspended in 0.5 N perchloric acid and incubated at 90 °C for 7 min in a constant temperature water bath, centrifuged and the supernatant was collected. The residue was washed twice with water and the supernatants were pooled to make up the volume to 15 ml. Equal volume of potassium hydroxide (1 N) was added to precipitate excess perchlorate as $KClO_4$. The extract was centrifuged at 5000 rpm for 15 min and the supernatant which contained DNA was collected.

ii). Estimation of DNA

To an appropriate amount of DNA extract, 4.0 ml of freshly prepared diphenylamine reagent (10 g of pure diphenylamine dissolved in 1 litre of glacial acetic acid and to it added 25 ml of conc. H_2SO_4). It was then heated in a boiling water bath for 10 min and cooled. The absorbance was calculated from a standard curve and was expressed in mg per 100 mg fresh weight.

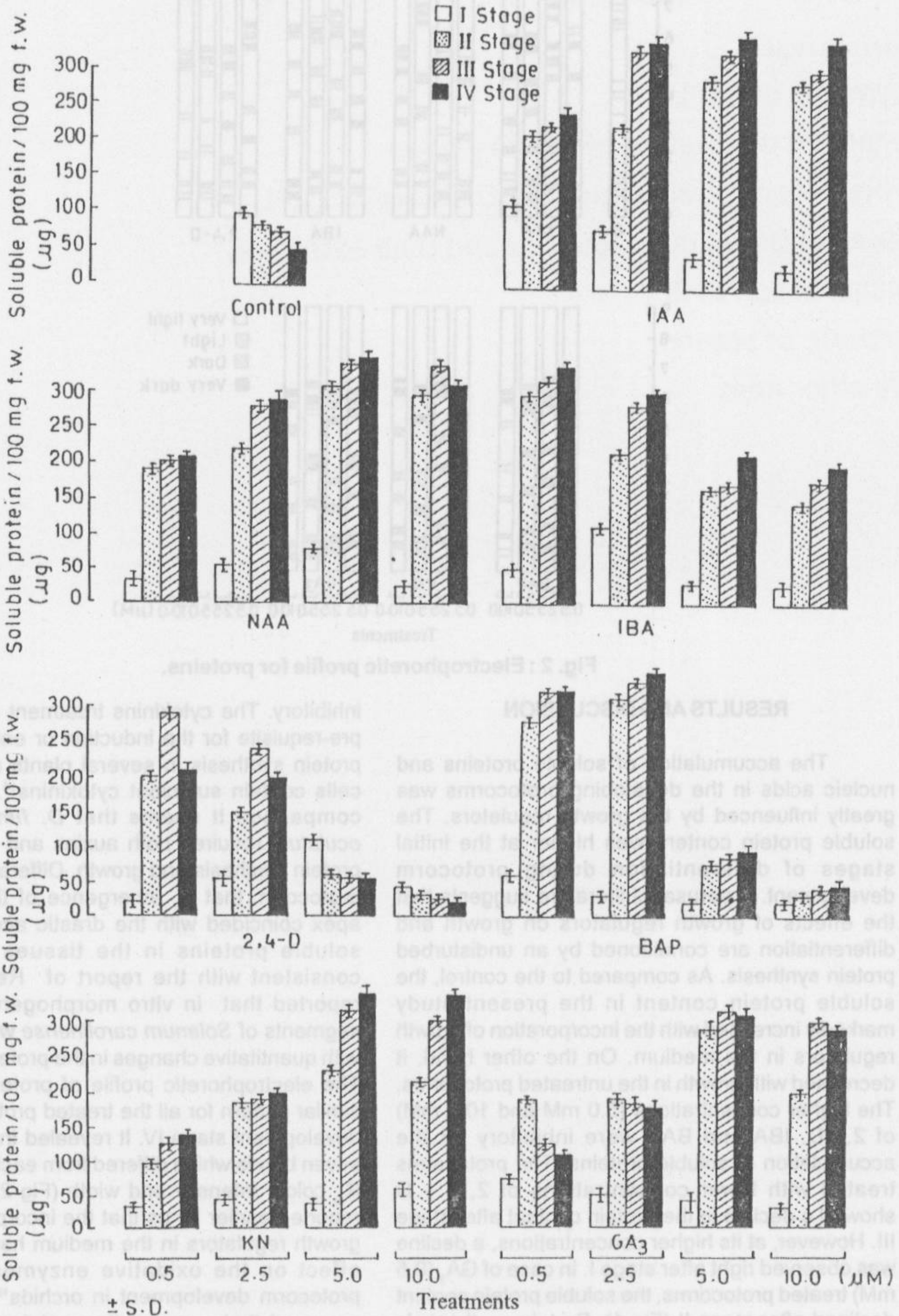

Fig. 1 : Effect of growth regulators on soluble protein content (µg/100 mg fresh wt.) at different stages of protocorm development of *Dendrobium fimbriatum* var. *oculatum*.

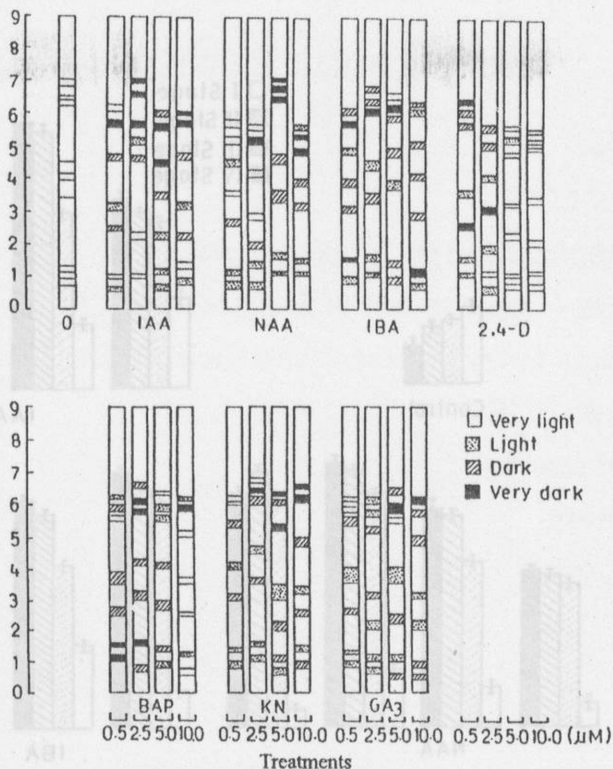

Fig. 2 : Electrophoretic profile for proteins.

RESULTS AND DISCUSSION

The accumulation of soluble proteins and nucleic acids in the developing protocorms was greatly influenced by the growth regulators. The soluble protein content was higher at the initial stages of differentiation during protocorm development. A perusal of literature suggests that the effects of growth regulators on growth and differentiation are conditioned by an undisturbed protein synthesis. As compared to the control, the soluble protein content in the present study markedly increased with the incorporation of growth regulators in the medium. On the other hand, it decreased with growth in the untreated protocorms. The higher concentrations (5.0 mM and 10.0 mM) of 2,4-D, IBA and BAP were inhibitory for the accumulation of soluble proteins. The protocorms treated with lower concentrations of 2, 4 – D showed a decline in the protein content after stage III. However, at its higher concentrations, a decline was observed right after stage I. In case of GA_3 (0.5 mM) treated protocorms, the soluble protein content declined after stage II (Fig 1). Protein synthesis is a pre- requisite for the growth to continue. The results show that the auxins stimulated the accumulation of soluble protein contents. However, higher concentrations of IBA and 2,4-D were inhibitory. The cytokinins treatment may not be a pre-requisite for the induction or enhancement of protein synthesis in several plants as most plant cells contain sufficient cytokinins[15]. However, in comparison it seems that *D. fimbriatum* var. *oculatum* requires both auxins and cytokinins for protein synthesis and growth. Differentiation of the protocorm, that is, emergence of the vegetative apex coincided with the drastic accumulation of soluble proteins in the tissues which is in consistent with the report of Reynolds[6] who reported that in vitro morphogenesis of stem segments of *Solanum carolinense* was associated with quantitative changes in the protein expression. The electrophoretic profile of proteins showed a similar pattern for all the treated protocorms at the development stage IV. It revealed the presence of seven bands which differed from each other in their R_m colour intensity and width (Fig 2). It has been reported earlier by us that the incorporation of the growth regulators in the medium has pronounced effect on the oxidative enzymes during the protocorm development in orchids[16,17]. It may be assumed that some of the proteins produced *de novo* as a result of the treatments belong to the oxidative enzyme pathway.

For any growth process to be sustained,

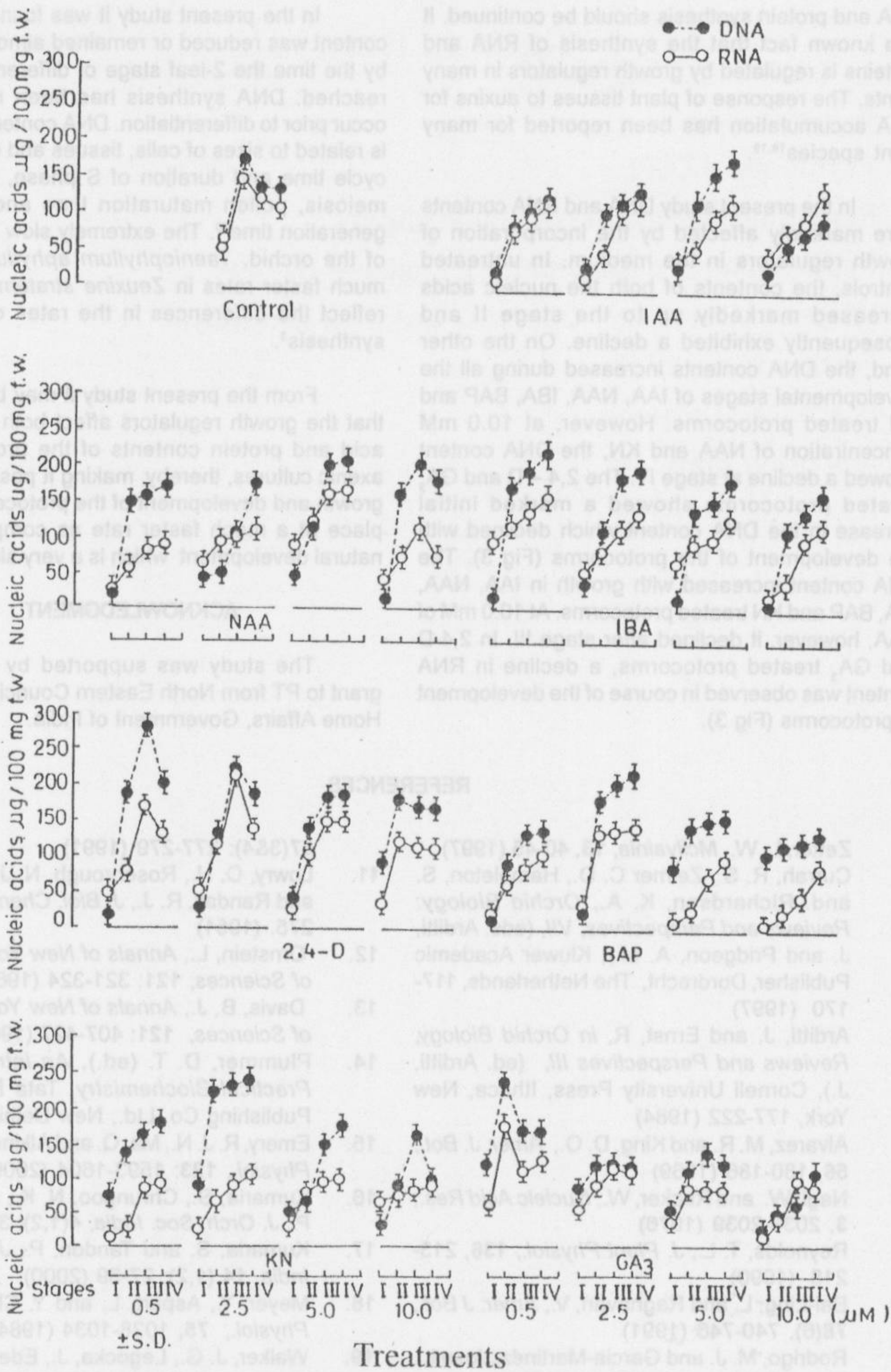

Fig. 3 : Effect of growth regulators on DNA and RNA accumulation (µg/100 mg fresh wt.) at different stages of protocorm development *Dendrobium fimbriatum* var. *oculatum*.

RNA and protein synthesis should be continued. It is a known fact that the synthesis of RNA and proteins is regulated by growth regulators in many plants. The response of plant tissues to auxins for RNA accumulation has been reported for many plant species[18,19].

In the present study DNA and RNA contents were markedly affected by the incorporation of growth regulators in the medium. In untreated controls, the contents of both the nucleic acids increased markedly up to the stage II and subsequently exhibited a decline. On the other hand, the DNA contents increased during all the developmental stages of IAA, NAA, IBA, BAP and KN treated protocorms. However, at 10.0 mM concentration of NAA and KN, the DNA content showed a decline at stage IV. The 2,4 – D and GA_3 treated protocorms showed a marked initial increase in the DNA content which declined with the development of the protocorms (Fig 3). The RNA content increased with growth in IAA, NAA, IBA, BAP and KN treated protocorms. At 10.0 mM of NAA, however, it declined after stage III. In 2,4-D and GA_3 treated protocorms, a decline in RNA content was observed in course of the development of protocorms (Fig 3).

In the present study it was found that DNA content was reduced or remained almost constant by the time the 2-leaf stage of differentiation was reached. DNA synthesis has been reported to occur prior to differentiation. DNA contents in plants is related to sizes of cells, tissues and organs, cell cycle time and duration of S phase, duration of meiosis, pollen maturation time and minimum generation time[20]. The extremely slow growth rate of the orchid, *Taeniophyllum aphyllum* and the much faster rates in *Zeuxine stratematica* might reflect the differences in the rates of the DNA synthesis[3].

From the present study it may be assumed that the growth regulators affect both the nucleic acid and protein contents of the protocorms in axenic cultures, thereby, making it possible for the growth and development of the protocorms to take place at a much faster rate as compared to its natural development which is a very slow process.

ACKNOWLEDGMENT

The study was supported by a research grant to PT from North Eastern Council, Ministry of Home Affairs, Government of India.

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