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Effect of phytohormones on nuclear RNA synthesis in germinating seeds of *Trigonella foenumgraeceum* and its callus

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Abstract. Treatment of *Trigonella foenumgraeceum* (fenugreek) seedlings with naphthalene acetic acid plus gibberellic acid enhanced the RNA synthesising capacity of nuclei isolated from the hypocotyl and cotyledonary regions. This increase was more pronounced in the nuclei from the hypocotyl region than from the cotyledonary region. *In vitro* addition of these phytohormones did not stimulate RNA synthesis by nuclei. The RNA synthesis by mitochondria was not affected by preincubating the seedlings with the hormones. The nuclei isolated from callus cultures of fenugreek hypocotyl treated with the hormone also showed increased RNA synthesis.

Keywords. Phytohormones; nuclear RNA synthesis; Trigonella foenumgraeceum; callus.

Introduction

Phytohormones like auxins, gibberellins and cytokinins play an important role in root and shoot formation from callus cultures and in seed germination. The biochemical events preceding differentiation are not clearly understood. However, it has been observed that during the development of embryo into shoot and root, enhanced synthesis of RNA and protein occurs. In many tissues, auxin and gibberellin treatment causes an increase in total RNA, mRNA and polysomes (Davies and Larkins, 1973; Thompson and Cleland, 1971). It has been demonstrated that these hormones enhance RNA polymerase activity (Johnson and Purves, 1970; McComb *et al*., 1970) through mediator molecules which bind to these hormones (Venis, 1973; Kende and Gardner, 1976; Jacobson 1977; Mennes *et al*., 1978). These reports suggest that differentiation may be a consequence of alterations in RNA and protein synthesis regulated by phytohormones. In this paper, we report preliminary studies on nuclear RNA synthesis in relation to exogenously added phytohormones during seed germination and callus growth.

Materials and methods

Chemicals

Ribonucleoside triphosphates (CTP, UTP, GTP and ATP), naphthalene acetic acid, gibberellic acid, 2,4-dichlorophenoxy acetic acid, kinetin, benzyl amino purine and indole acetic acid, tris and 2-mercaptoethanol were purchased from Sigma Chemical co., St Louis, MO, USA and [8-^{3}H] ATP (24 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England; all other chemicals were of the analytical reagent grade.

Plant material

Fenugreek seeds purchased from local market were pretreated with 0·1 % $HgCl_2$ for 20 min., washed with sterile water and planted in sterile trays with moist filter paper and germinated in the dark. After germination, seedlings were removed under sterile conditions and incubated with a solution containing 1 mM naphthalene acetic acid (NAA) and 0·1 mM gibberellic acid (GA_3) at pH 6·5 for 16h.

Callus growth and subculture

Fenugreek callus was initiated and maintained in Murashige and Skoog's medium (MS medium, Murashige and Skoog, 1962). MS medium was tested in combination with various growth hormones like indole acetic acid (IAA), kinetin, 2,4-dichlorophenoxy acetic acid (2,4-D), coconut milk, benzyl amino purine (BAP) and naphthalene acetic acid (NAA). Maximum growth was obtained in MS supplemented with 2 parts per million (ppm) NAA and 2 ppm BAP. The callus formation was not satisfactory in MS with 2,4-D and/or kinetin or IAA. Though fairly good growth was observed in a medium supplemented with coconut milk, the callus turned brown at an early stage. In the presence of NAA and BAP, the hypocotyls became swollen within a week and callused within 15–20 days. On subculturing, the callus showed a lag phase and reached mid-exponential phase in about 2 weeks and stationary phase in 4 weeks.

The seeds were germinated under sterile conditions (pretreated with 0·1% $HgCl_2$) and grown for three days. The hypocotyls were cut into 1 cm pieces and were inoculated in 100 ml flasks containing 20 ml. medium. The callus was subcultured in fresh medium once in three weeks.

Isolation and purification of nuclei

The isolation of nuclei was done by a modification of Hamilton's method (Hamilton *et al*., 1972). The tissue was homogenised in grinding medium (50 mM tris-HCl buffer pH 8 0, 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$ and 500 mM sucrose) using a Waring blendor for 1 min. 30 sec. The homogenate was filtered through four layers of cheese-cloth and the residue was homogenised twice as above. The homogenates were pooled and allowed to stand for 1 h to eliminate most of the starch grains. The supernatant was decanted and centrifuged at 800 g for 15 min. The pellet was washed twice with grinding medium and purified further, by the method of Huang and Bonner (1962) with a slight modification. The pellet was suspended in 7 ml of grinding medium and layered over 23 ml of the same medium containing 2 M

sucrose, the upper 2/3rd portion was stirred well to form a rough gradient and centrifuged at 51,000 g for 3 h in an SW 25 rotor, Beckman L2–50 ultracentrifuge. Under these conditions, the nuclei were sedimented whereas the plastids and other contaminants remained on the top. The nuclear pellet suspended in grinding medium was assayed for RNA polymerase activity.

Isolation and purification of mitochondria

Mitochondria were isolated by slight modification of the method prescribed by Chakrabarti *et al*. (1972). The homogenate fraction was centrifuged at 800 g followed by three centrifugations of the supernatant fraction at 3,000 g for 15 min and each time the pellet was discarded. The mitochondria were sedimented by centrifuging the supernatant fraction at 10,000 g for 15 min and washed twice with grinding medium. The mitochondria were purified by suspending the pellet in 1 ml of grinding medium, layering over a discontinuous gradient of the same medium containing 2.4 M (3 ml), 1 M (1 ml) and 0.75 M (1 ml) sucrose, centrifuging at 100,000 g for 20 min in an SW 50 rotor (Richter and Lipmann, 1970). The mitochondria were collected from the 1 M sucrose region and assayed for RNA polymerase activity.

RNA polymerase assay

The assay mixture (0·25 ml) contained tris-HCl buffer pH 8·0, 20 μmol; 2-mercaptoethanol, 10 μmol; $MgCl_2$, 1 μmol; $MnCl_2$. 0·25 μmo1, UTP, GTP and CTP 0·1 μmol each; [^{3}H]-ATP (24 Ci/mmol), 1 μCi); nuclear suspension, 0·1 ml The reaction was carried out at 37° C for 15 min and terminated by addition of 2 ml of 10% trichloroacetic acid (Duda and Cherry, 1971). The precipitates were collected on Whatman No. 3MM filter paper discs and washed with 10% and 5% trichloroacetic acid; ethanol-ether mixture (1 : 1) and finally by ether dried and radioactivity measured in an LS-100 liquid scintillation spectrometer using 10 ml toluene-based scintillation fluid.

Results

RNA synthesis in the fenugreek seedlings

The nuclei showed maximal RNA synthesis after three days of germination in control as well as hormone-treated seedlings (figure 1). After 72 h of germination, the activity slowly declined. No activity could be detected in ungerminated, water-imbibed seeds. Three-day old seedlings were used for further experiments. Nuclear RNA synthesis increased linearly for 15 min (figure 2).

Effect of NAA and GA_3 on nuclear RNA synthesis

NAA and/or GA_3 treatment enhanced total nuclear RNA synthesis in the seedlings. It was observed that 1 mM NAA and 0·1 mM GA_3 elicited the optimal response (table 1). When both the hormones were used together (1mM NAA with 0·1 mM GA_3), maximum RNA synthesis was obtained.

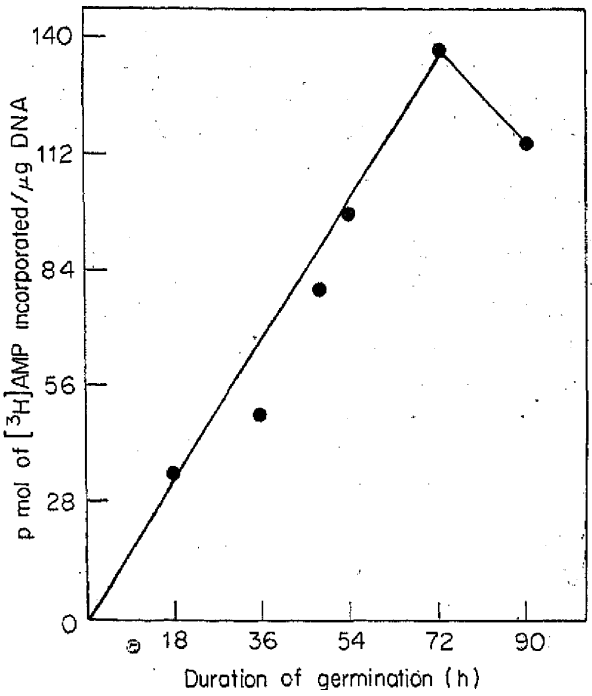

Figure 1. RNA synthesis by nuclei isolated, from *T. foenumgraeceum* seedlings. The nuclei were isolated from seeds at different stages of germination and RNA synthesis was measured *in vitro* as described in methods. The nuclei from ungerminated (pre-soaked for 4 h) seeds did not show any RNA synthetic activity. Various points on the X-axis correspond to the time at which the seedlings were processed.

The nuclei obtained from hypocotyl region elicited a 5-fold increase on treatment with NAA and GA_3, whereas those from cotyledons showed only a 3-fold enhancement in RNA synthetic activity.

The hormones did not bring about any change in RNA synthesis when added to the reaction mixture (table 2). The mitochondrial RNA synthesis was not affected by the hormone treatment.

Callus growth in relation to RNA synthesis

The maximum activity was shown by the callus at the end of the exponential phase. However, the *in vitro* RNA synthetic activity of the nuclei declined when isolated, from three weeks old callus tissue, though the dry weight remained the same for another 5–7 days (figure 3).

Effect of phytohormones on RNA synthesis of nuclei isolated from fenugreek callus cultures

As in the case of the seedlings, nuclei isolated from the callus elicited a 5-fold increase in RNA synthesis on treatment with the hormones (table 3).

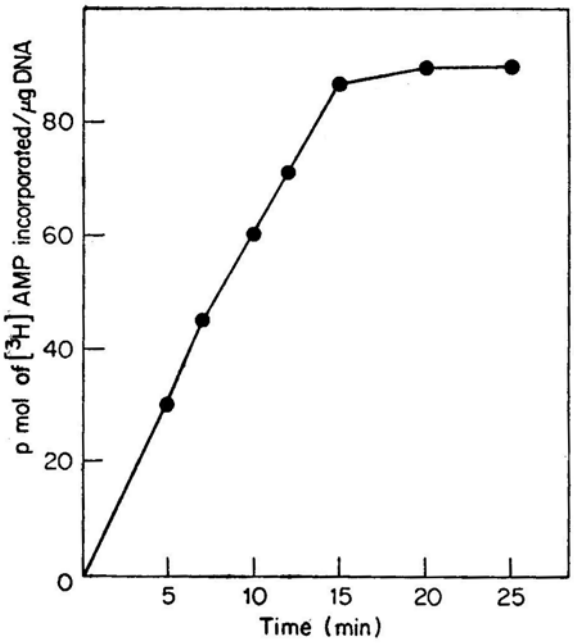

Figure 2. Time course of RNA synthesis by nuclei from *T. foenumgraeceum* seedlings
RNA synthesis by the nuclei *in vitro* was measured at different time intervals.

Table 1. Effect of phytohormones on nuclear RNA-polymerases from *T. foenum-graeceum.*

Treatment		pmol of [^{3}H] AMP incorporated/ µg DNA
None		140
NAA	10 mM	50
	1 mM	300
	0·1 mM	180
GA_3	1 mM	40
	0·1 mM	430
	0·01 mM	280

NAA = Naphthalene acetic acid.
GA_3 = Gibberellic $acid_3$.

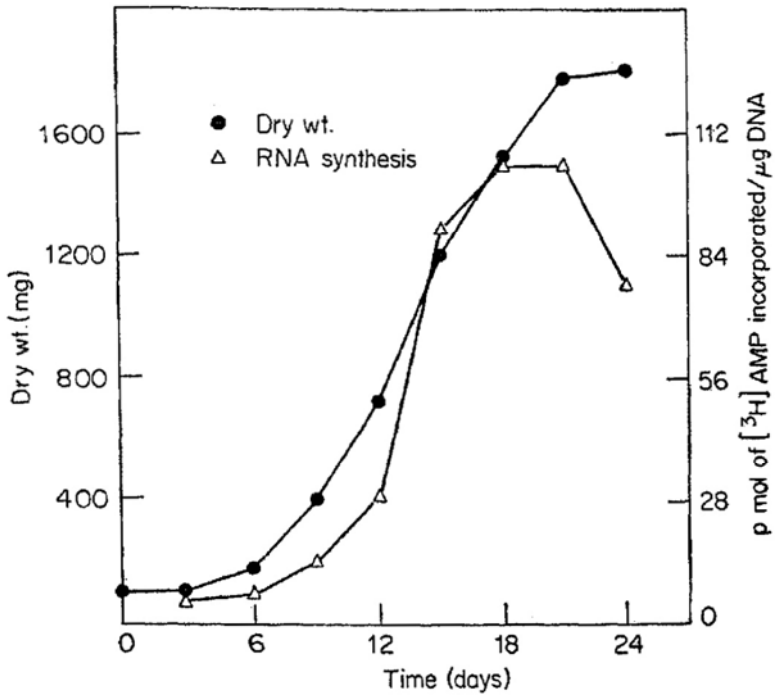

Figure 3. RNA synthesis by nuclei from *T. foenumgraeceum* callus tissue.

Table 2. Effect of NAA in combination with GA_3, on nuclear RNA polymerases from *T. foenumgraeceum* hypocotyls and cotyledons.

Tissue	Treatment	pmol of (^{3}H) AMP incorporated/μg DNA	Fold increase
Hypocotyls	(*a*) Nil	140	
	(*b*) + Hormones *in vitro*	130	
	(*c*) Pretreated	750	5
Cotyledons	(*a*) Nil	27	
	(*b*) + Hormones *in vitro*	28	
	(*c*) Pretreated	86	3

Cotyledons and hypocotyls were treated with mM NAA and 0·1 mM GA_3 (pH6·5) for 16h. The nuclei were is olated from control and treated tissues. The RNA polymerase was assayed as described in the text.

a. Control nuclei assayed in the absence of the hormones in the reaction mixture.
b. Control nuclei + 1 mM NAA and 0·1 mM GA_3 included in the reaction mixture during the assay.
c. Nuclei from treated tissue assayed in the absence of the hormones in the reaction mixture.

Table 3. Effect of phytohormones on nuclear RNA polymerases from *T. foenum-graeceum* callus cultures.

Tissue	pmol of [^{3}H]-AMP incorpo-rated/μg DNA	Fold increase
Control	105	
Pretreated	520	5

About 100 g callus (3 weeks old) was pre-treated with 1 mM NAA and 0·1 mM GA_3 as in the case of seedlings. Nuclei were isolated from control as well as treated callus and RNA polymerases were assayed as described in the text.

Discussion

The present study shows that *T. foenumgraeceum* seedlings pretreated with an auxin, in combination with a gibberellin, exhibit enhanced nuclear RNA synthesis which is more pronounced in the hypocotyl region than in the cotyledonary region, It has long been known that, upon treatment with phytohormones, the nucleic acid content of a number of plant tissues increases (Key, 1969; Maheswari *et al*., 1966). Chen *et al*. (1975) have shown that nuclei from 2,4-D (2·5 mM)-treated hypocotyls of soyabean contain about 2·5 times as much RNA polymerase I activity as nuclei from untreated tissues. Our results are in agreement with those of Hou and Pillay (1975) who found that GA also markedly enhances RNA synthesis in chromatin preparations of treated soyabean hypocotyls. Earlier workers (Rijvan and Parkash, 1970) have reported that stimulation of growth in fenugreek seedlings by kinetin is associated with a large increase in RNA synthesis followed by enhanced protein synthesis. Fenugreek seedlings showed more enhancement in RNA synthetic activity when NAA was used in combination with GA_3 (5-fold) than when the hormones were used separately (2-fold with NAA and 3-fold with GA_3). It is interesting to note that a similar enhancement was elicited by nuclei isolated from fenugreek callus cultures, after treatment with NAA and GA_3.

However, addition of hormones to the isolated nuclei did not affect the RNA polymerase activity. This may reflect the requirement of a receptor molecule which may have been leached out during the isolation of nuclei carried out in the absence of hormones. This is in agreement with some of the earlier work (Matthysse and Phillips, 1969).

The enhancement in the RNA synthetic activity could be brought about either by the increased template availability or due to an enhanced synthesi and/or activity of nuclear RNA polymerases. Our results do not distinguish between these possibilitites Further studies are in progress.

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