Phytochemistry, 1972, Vol. 11, pp. 3181 to 3185. Pergamon Press. Printed in England.

INHIBITION OF GERMINATION IN *CICER ARIETINUM**

A. K. SRIVASTAVA, S. AZHAR and C. R. KRISHNA MURTI
Central Drug Research Institute, Lucknow, India

(*Received* 3 *January* 1972. *Accepted* 23 *May* 1972)

Key Word Index—*Cicer arietinum*; Leguminosae; inhibition of germination; cycloheximide; 8-azaadenine; reversal of germination inhibition; cyclic AMP; adenosine.

Abstract—8-Azadenine, cycloheximide, DL-ethionine and *p*-fluorophenylalanine inhibited the germination of *Cicer arietinum*. Inhibition by 8-azaadenine was reversed by either adenosine, cyclic-3′,5′-AMP or indole-3-acetic acid. Inhibition by cycloheximide was not reversible by any of the agents tested. Degradation of reserve proteins, polysaccharides and phosphates which occurred during germination was also inhibited by these agents. When seeds were exposed to inhibitors during imbibition, the induction of proteases, amylase, phosphatase and peroxidase activities that occurred during germination was inhibited, indicating that these enzymes appeared in seedlings by *de dovo* synthesis.

INTRODUCTION

THERE is evidence that the loss of proteins, polysaccharides and bound phosphates of *Cicer arietinum* during germination is accompanied by activation of enzymes which mediate the degradation of the polymers.[1] In order to gain some insight into the regulatory mechanism of such activity, the effect of a number of well known protein inhibitors have been studied during the germination of this legume.

TABLE 1. EFFECT OF INHIBITORS OF MACROMOLECULAR SYNTHESIS ON THE GERMINATION OF *C. arietinum* SEEDS

Addition to germinating medium	Embryo length (cm) at following periods of germination (hr) 48	72	96	120	144
None	3·0	5·0	10·0	13·1	15·2
p-Fluorophenylalanine* (10^{-3} M)	1·8	3·0	5·0	8·0	8·8
p-Fluorophenylalanine† (10^{-3} M)	1·0	2·5	4·5	7·0	7·5
None	2·8	5·5	9·0	14·0	15·5
DL-Ethionine (10^{-3} M)	2·0	4·0	6·0	7·0	7·5
8-Azaadenine (10^{-3} M)	0	0·1	0·1	0·2	0·3
Cycloheximide (10^{-3} M)	0	0	0	0	0

* Seed imbibed for 5 hr.
† Present throughout germination.

RESULTS

Effect of Inhibitors on Embryo Growth

The effect of a number of inhibitors on the germination of *C. arietinum* are shown in Table 1. In the presence of 8-azaadenine (10^{-3} M) or cycloheximide (10^{-3} M) embryo

* Communication No. 1697 from the Central Drug Research Institute, Lucknow, India.

[1] S. AZHAR, A. K. SRIVASTAVA and C. R. KRISHNA MURTI, *Phytochem.* **11**, 3173 (1972).

formation was completely inhibited. Cycloheximide (Fig. 1) and 8-azaadenine, both at 10^{-3} M, were shown to inhibit further germination when added at various stages in the process. At the same concentration, DL-ethionine and *p*-fluorophenylalanine were less effective (unpublished data).

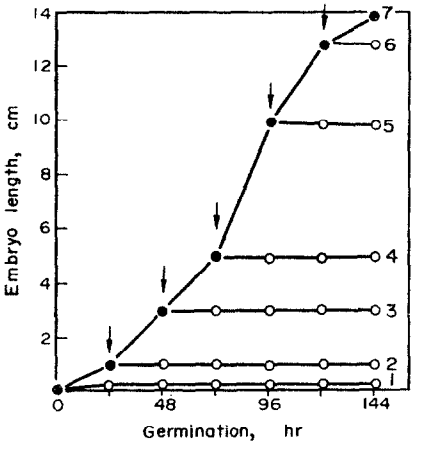

FIG. 1. EFFECT OF ADDING 1×10^{-3} M CYCLOHEXIMIDE AT DIFFERENT STAGES OF GERMINATION OF *C. arietinum* SEEDS.

At the time indicated by arrowseeds germinating in aqueous medium were transferred to 1×10^{-3} M cycloheximide. Each point on the curve is the mean embryo length of 8–10 seeds.

Reversal of Inhibition by Adenosine

Adenosine 10^{-3} M, IAA and *c*-AMP partially reversed the inhibitory effect of 8-azaadenine (Table 2). Seeds imbibed prior to germination for 6 hr in 10^{-3} M azaadenine germinated very slowly. Such seeds, however, on washing and further exposure to adenosine, IAA or *c*-AMP for 6 hr readily germinated. The inhibitory effect of cycloheximide could not be reversed by either IAA (10^{-4} M) or *c*-AMP (10^{-4} M).

TABLE 2. REVERSAL OF 8-AZADENINE-INHIBITED GERMINATION OF *C. arietinum* SEEDS BY ADENOSINE, IAA AND *c*-AMP

Additions to germinating medium	Embryo length (cm) at following periods of germination (hr)				
	48	72	96	120	144
None	2·0	4·0	6·2	9·0	11·2
8-Azaadenine (10^{-3} M)	0	0·1	0·2	0·2	0·3
IAA (10^{-5} M)	2·0	4·2	6·4	8·9	10·9
c-AMP (10^{-5} M)	2·0	4·1	6·4	8·9	11·0
Adenosine (10^{-3} M)	2·0	4·0	6·1	9·0	11·1
Cycloheximide (10^{-3} M)	0	0	0	0	0
8-Azaadenine (10^{-3} M) + IAA (10^{-5} M)	0·7	2·2	3·2	4·8	5·9
8-Azaadenine (10^{-3} M) + *c*-AMP (10^{-5} M)	0·4	1·3	1·7	3·2	5·0
8-Azaadenine (10^{-3} M) + adenosine (10^{-3} M)	1·0	2·0	2·9	4·1	5·1

c-AMP, Cyclic 3′,5′-adenosine monophosphate: IAA, Indole-3-acetic acid.

Effect of Inhibitors on the Degradation of Food Reserves Proteins

Cycloheximide and 8-azaadenine completely blocked the degradation of proteins and the increase in free aminoacids; DL-ethionine was less effective. Patterns of protease activity in untreated and inhibitor-treated seeds are given in Table 3.

TABLE 3. EFFECT OF INHIBITORS OF MACROMOLECULAR SYNTHESIS ON THE LEVELS OF PROTEASE AND ACID PHOSPHATASE DURING THE GERMINATION OF *C. arietinum* SEEDS

	Specific activity (units/mg protein)							
	Protease				Acid phosphatase			
Germination (hr)	None	8 Aza-adenine	Cyclo-heximide	DL-Ethi-onine	None	8 Aza-adenine	Cyclo-heximide	DL-Ethi-onine
0	38	38	39	40	0·20	0·20	0·20	0·20
24	55	40	40	40	0·30	0·21	0·19	0·23
48	73	50	43	44	1·40	0·20	0·20	0·90
72	117	20	41	40	1·80	0·20	0·21	1·20
96	130	18	20	43	2·40	0·21	0·20	1·40
120	150	16	18	41	3·80	0·21	0·19	1·10

Inhibitiors concentration were 10^{-3} M.

Starch

The mobilization of starch was completely inhibited by cycloheximide and 8-azaadenine and partially by DL-ethionine. Changes in the activities of α-amylase and starch phosphorylase under the influence of the inhibitors are given in Table 4. The induction of α-amylase during germination was completely blocked by cycloheximide or 8-azaadenine and partially by DL-ethionine. The three reagents partly inhibited the induction of starch phosphorylase activity.

TABLE 4. EFFECT OF INHIBITORS OF MACROMOLECULAR SYNTHESIS ON THE LEVELS OF α-AMYLASE AND STARCH PHOSPHORYLASE DURING THE GERMINATION OF *C. arietinum* SEEDS

	Specific activity (units/mg protein)							
	Starch phosphorylase				α-Amylase			
Germination (hr)	None	8 Aza-adenine	Cyclo-heximide	DL-Ethionine	None	8 Azadenine	Cyclo-heximide	DL-Ethionine
0	6·0	5·9	5·9	6·1	0·12	0·12	0·11	0·12
24	11·0	7·0	7·6	8·0	0·18	0·13	0·13	0·14
48	15·0	10·0	10·5	9·7	0·24	0·11	0·12	0·15
72	18·0	12·1	11·0	10·3	0·48	0·16	0·10	0·30
96	20·0	13·4	15·0	13·2	0·91	0·12	0·12	0·61
120	25·0	14·8	16·8	15·1	1·20	0·10	0·10	0·53

Inhibitors concentration were 10^{-3} M.

Phosphates

The release of P_i from bound phosphates during germination was promptly arrested by 8-azaadenine, cycloheximide and DL-ethionine; their effect on the induction of acid phosphatase is shown in Table 3.

Effect on Peroxidase

Large increases in peroxidase activity which occur during germination were partly prevented by 8-azaadenine and cycloheximide (Table 5). DL-Ethionine exerted a smaller inhibitory effect.

TABLE 5. EFFECT OF INHIBITORS OF MACROMOLECULAR SYNTHESIS ON THE LEVELS OF PEROXIDASE DURING THE GERMINATION OF *C. arietinum* SEEDS

Addition to germinating medium	Specific activity (units/mg protein) at following periods of germination (hr)					
	0	24	48	72	96	120
None	0·15	7·1	200	500	804	1080
8 Azaadenine (10^{-3} M)	0·14	0·4	3·9	11	10	39
Cycloheximide (10^{-3} M)	0·12	0·2	4·8	5·5	12	20
DL-Ethionine (10^{-3} M)	0·12	2·0	70	242	220	131

DISCUSSION

Not surprisingly, 8-azaadenine and cycloheximide are very potent inhibitors of the germination of *C. arietinum* and of many metabolic processes that accompany germination. The inhibitory effect of 8-azadenine on seed germination could be partially reversed by adenosine, IAA and *c*-AMP whereas neither IAA nor *c*-AMP were able to reverse the inhibitory effect of cycloheximide. The less pronounced effects observed with DL-ethionine or *p*-fluorophenylalanine may be due to the fact that primary regulatory events are not completely inhibited by these agents or due to the gradual detoxication of these analogues by the embryo. The results of the present study generally support the view of Ingle[2] that growth and development are regulated by nucleic acids which are elaborated in the early imbibition phase and which lead to the appearance concurrently of proteins required for both degradative and biosynthetic activities. The effect of inhibitors on the induction of peroxidase was less pronounced.

The activity of ribosomes to incorporate exogenously supplied amino acids into proteins has been shown to increase with the period of germination of *C. arietinum*.[3] Among the new proteins are the various hydrolytic enzymes synthesized in order to degrade the reserve polymers and supply new building units for the developing tissue. The fact that the addition of cycloheximide arrests further development when added at various stages of germination lends support to the idea that regulation of the developmental processes is mediated by protein synthesized during germination. Induction of α-amylase and ATPase in peas,[4] protease activity in cotton seed[5] and glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in peas[6] during germination are all now recognized as due to *de novo* synthesis. The present results with protease, α-amylase and acid phosphatases also show that the increase in activity is due to net protein synthesis.

[2] J. INGLE, *Phytochem.* **6,** 456 (1967).
[3] S. M. HADI and C. R. KRISHNA MURTI, *Ind. J. Biochem.* **4,** 1 (1967).
[4] J. L. YOUNG and J. E. VARNER, *Arch. Biochem. Biophys.* **84,** 71 (1959).
[5] J. N. IHLE and L. DURE, *Biochem. Biophys. Res. Comm.* **36,** 705 (1969).
[6] A. P. BROWN and J. L. WRAY, *Biochem. J.*, **108,** 437 (1968).

Evidence has been obtained to indicate that the synthesis of cyclic AMP is stimulated several fold during the imbibition of *C. arietinum* seeds; the activity, however, declines as germination proceeds (Azhar *et al.*, unpublished observations). It is likely that *c*-AMP is in some unknown manner linked with the mechanism of regulation of protein synthesis in the early phase of germination. The nature of the macromolecules modulated by the nucleotide in the plant tissue is unknown. Indole-3-acetic acid stimulates the synthesis of *c*-AMP in *C. arietinum* seedlings.[7] It can be postulated that a repressor system which keeps adenyl cyclase in a relatively less active form during dormancy has to be removed from the site of action, and possibly what happens during seed imbibition is the synthesis of an enzyme that inactivates the repressor. Cycloheximide and 8-azaadenine perhaps interfere with the synthesis of this derepressing enzyme.

EXPERIMENTAL

Germination of *C. arietinum* was carried out as described earlier.[1] The inhibitors in appropriate concentration were added to the water used for imbibition of the seeds or were present in the aqueous environment in which imbibed seeds were maintained during germination. In testing the effect of inhibitors at different stages of germination, seeds were sampled from the germination medium and soaked for 5 hr in a solution of the inhibitor.

Samples of germinated seeds were used for measuring embryo length, chemical composition and enzyme activity. The plant tissues were processed for estimation of free sugars, amino acids, polysaccharides proteins and phosphorus as described earlier.[1]

Enzyme assays. Preparation of enzyme extracts has been described earlier.[1] Seeds were not fractionated into cotyledons and seedlings before homogenization. Protease, α-amylase (α-1,4-glucan-4-glucanohydrolase E.C. 3.2.1.1), starch phosphorylase (α-1,4 glucan:orthophosphate glucosyl transferase E.C. 2.4.1.1) and acid phosphatase (orthophosphoric monoester phosphohydrolase E.C. 3.1.3.2) were assayed by methods referred to in the preceding paper.[1] Units of enzyme activity were defined as the amount which gave an Absorbance increase of 0·001 in 1 hr (protease), which liberated 0·1 mg reducing group (calculated as maltose) in 10 min (α-amylase), which liberated 100 mμmol P_i in 10 min (starch phosphorylase) and which liberated 1 μmol P_i in 15 min (acid phosphatase).

Peroxidase (Donor:hydrogen peroxide:oxidoreductase, E.C. 1.11.1.7) was assayed by a modification of the procedure of Evans.[8] The reaction mixture contained 100 μmol phosphate buffer pH 6·2, 15 μmol H_2O_2 and 0·1 ml enzyme appropriately diluted in a final vol. of 3·0 ml. The reaction was initiated by the addition 60 μmol of guaiacol. The peroxidation of guaiacol to tetraguaiacol was calculated from an E_{470nm} value of 26·6 cm^{-1} mM^{-1}. A unit of activity was defined as the amount of enzyme required to liberate 1 nmol tetraguaiacol in 1 min.

Chemical estimations. Amino acids, protein, total free sugars, reducing sugars and phosphorus were determined by methods referred to in the preceding paper.

[7] S. Azhar and C. R. Krishna Murti, *Biochem. Biophys. Res. Commun.* **43**, 58 (1971).
[8] J. J. Evans, *Plant Physiol.* **43**, 1037 (1968).