Plant Physiol. (1990) 93, 1011-1016
0032-0889/90/93/1011/06/$01.00/0

Received for publication March 31, 1989
Accepted November 15, 1989

Influence of Endogenous Cytokinins on Reverse Mobilization in Cotyledons of *Cicer arietinum* L.

Reproduction of Endogenous Levels of Total Cytokinins, Zeatin, Zeatin Riboside, and Their Corresponding Glucosides

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ABSTRACT

The embryonic axis plays an essential role in the mobilization of the main reserves of the cotyledons of seeds of *Cicer arietinum* L. cv Castellana. This control by the axis of the metabolism of the storage products of the cotyledons largely takes place through the cytokinins, which are transported from the embryonic axis to the cotyledons where the mobilization of reserves begins. The principal regulatory role of the endogenous cytokinins concerns the metabolism of carbohydrates and proteins; there is less influence on lipid metabolism. However, each cytokinin seems to have a different role in the mobilization processes. The glucosides, glucosyl zeatin riboside, and glucosyl zeatin act only as storage forms of the hormones. Zeatin riboside affects mainly the mobilization of carbohydrates and has less effect on protein mobilization. Zeatin regulates both the mobilization of carbohydrates and that of proteins and is more marked in the latter case.

Most research on the control of reserve mobilization has been conducted in cereals (3, 31). This is possibly due to the difficulty of isolating component tissues and organs of other species for investigation, a process which is straightforward with cereal grains as a result of the spatial separation of the different tissues.

Reserve mobilization in cereal seeds appears to be controlled directly by the embryo, via the production of gibberellines which initiate the synthesis and secretion of hydrolytic enzymes from the aleurone layer into the endosperm (31).

In dicotyledoneous plants, the regulation of food reserve mobilization is less well understood (11) and many conflicting results exist in the literature concerning the role of the embryo or embryonic axis in the breakdown of seed reserves (3, 22, 26).

Stimulated, no doubt, by the elucidation of the mechanism in cereals, several attemps have been made to demonstrate that a factor from the embryo or axis is also implicated in other seeds. This has been approached in two ways. First, the promotive capacity of extracts or diffusates of embryos and axes has been investigated; second, it has been argued that if a known hormone such as gibberellin or cytokinin can replace the promotive effect of the embryo or axis, this raises the possibility that an endogenous hormone might be involved (3).

Numerous workers have confirmed the role of cytokinins in reserve mobilization in cotyledons from dicotyledoneous seeds (9, 17); however, in most studies the endogenous level of these hormones have not been taken into account, and the application of endogenous hormone could alter the response.

The aim of the present work was to provide a clearer idea of the role of cytokinins in reserve mobilization, carrying out all studies after reproducing the endogenous level of cytokinins. To do so we used chick-pea seeds from which the embryonic axis had been removed, so that we knew that there would be no detectable cytokinins (16). Then, by reproducing the endogenous levels of the different cytokinins present under normal conditions one can gain a clear idea of the role played by each of these substances in reserve mobilization.

MATERIALS AND METHODS

Plant Material

Two kind of seeds were used: *Cicer arietinum* L. cv Castellana (chick-pea) seeds for the evaluation of cytokinins and *Cucumis sativus* L. cv Calahorra (cucumber) seeds for the bioassay.

The chick-pea seeds were germinated and grown on a glass plate covered with filter paper, in darkness at 25°C with 80% RH. The cucumber seeds were germinated in the same way at 28°C.

Purification and Quantitation of Cytokinins

Extraction was carried out as described by Smith and Van Staden (23). The extracts eluted from the Dowex 50W × 8 column were dried and redisolved in 1 mL of 80% ethanol and the sample spotted onto a Silicagel 60 G plates. The plates were developed with isopropanol:ammonia:water (10:1:1) (12).

The chromatograms were later divided into 11 bands. Once each band had been eluted in 80% ethanol, filtered, and dried, it was used for the bioassay which was performed using the cucumber cotyledons (8). The bands exhibiting cytokinin activity were used for analysis by HPLC.

Chromatographic analysis was performed by reverse phase HPLC using a Varian 5060 liquid chromatograph equipped with a Vista CDS-401 computer. Conditions were as follows: column, 290 × 4 mm i.d. packed with Micro-Pack MCH-5; mobile phase, metanol: water (40:60 v/v) to (54:46 v/v) using a linear gradient for 12 min; flow rate 0.5 mL/min; temperature, 30°C; pressure, 148 atm; UV detector set at 254 nm (15).

The retention time was determined by use of cytokinin standards obtained from Sigma Chemical Co. under the conditions described above. Cytokinin calibration curves were calculated from peak height obtained with UV absortion at 254 nm. We calculated the amount of each cytokinin isolated by the height of the absortion peak at 254 nm.

Artificial Reproduction of Endogenous Levels of Cytokinins in Excised Cotiledons

For this study the same amount of plant material (50 seeds) was used as for the extraction processes. The seeds were germinated as usual. After 6 h of germination the axis was substituted by an agar block (1% agar) into which the total amount of cytokinin extracted from the same number of cotyledons of 12 h germinated seeds was injected (dissolved in water) with a microsyringe. After several checks we found that there is always a period of 6 h before the cytokinins injected into the agar have passed into the cotyledons. A second lot of seeds was germinated, and then the axes removed after 12 h of germination; cytokinins were extracted from the same number of 18 h germinated seed cotyledons and injected into the agar block. This procedure was repeated at 18 h of germination; in this case, injecting, after removing the axis, the cytokinins extracted from seed cotyledons germinated for 24 h. Since after 24 h of germination the maximum amount of cytokinin is reached in the cotyledons (16), from this moment it was not necessary to inject cytokinin. Endogenous cytokinins were extracted after 24, 36, 48, 72, 96, and 120 h of germination.

All the experiments were conducted three times.

Artificial Reproduction of Endogenous Levels of Glucosyl Zeatin Riboside, Glucosyl Zeatin, Zeatin Riboside, and Zeatin in Excised Cotyledons

Endogenous levels of ZROG,[1] ZOG, ZR, and Z were reproduced according to the method for total cytokinins mentioned above.

In the case of Z, it was only necessary to inject at 6 h germination to reach the normal endogenous levels obtained at the remaining times studied.

Reserve Mobilization in Chick-Pea

All studies were performed with both cotyledons collected during the germination of intact seeds and with cotyledons obtained from growing seeds without their axes (with and without reproduction of their endogenous levels of cytokinins).

[1] Abbreviations: Z, *trans*-zeatin; ZR, *trans*-zeatin riboside; ZOG, *trans*-zeatin-O-glucoside; ZROG, *trans*-zeatin riboside-O-glucoside.

Total carbohydrates were determined by the sulfuric acid-phenol colorimetric method (7). For extraction of lipids, the gravimetric method of Bligh and Dyer (4) was used. For extraction of soluble sugars, the residue resulting from lipid extraction was employed. To this residue 80% ethanol was added and after shaking for a few minutes the mixture was left to settle for 45 min., after which it was centrifuged at 2000*g* for 20 min. The supernatant was recovered and the above-described steps were repeated twice on the residue. The supernatants from the three extractions were pooled and concentrated in a Rotavapor down to a total volume of 15 mL, where the evaluation of soluble sugars was performed (18, 23, 24). For extraction of protein (5) the residue obtained, once dry, was dissolved with 0.05 N NaOH at 37°C for 4 h (2). Quantitative measurement was performed according to the method of Bradford (6). Total amylase activity was assayed according to the method described by Metivier and Paulilo (17). For the study of protease activity, the enzymatic extract was prepared according to the method described by Metivier and Paulilo (17). Determination of protease activity was performed by two methods: (a) measuring amino acid release (32) and performing the colorimetric method proposed by Yemm and Cocking (30); (b) measuring peptide release (32), measuring directly the absorbance, at 280 nm, of the supernatant obtained from the centrifugation (13).

RESULTS AND DISCUSSION

Artificial Reproduction of Endogenous Levels of Total Cytokinins in Excised Cotyledons

To reproduce the levels of total cytokinins (Fig. 1), it was necessary to inject different amounts of the cytokinins previously extracted at 6, 12, and 18 h of incubation. The results show that the levels of cytokinins in the cotyledons were similar to those observed in cotyledons of germinating seeds.

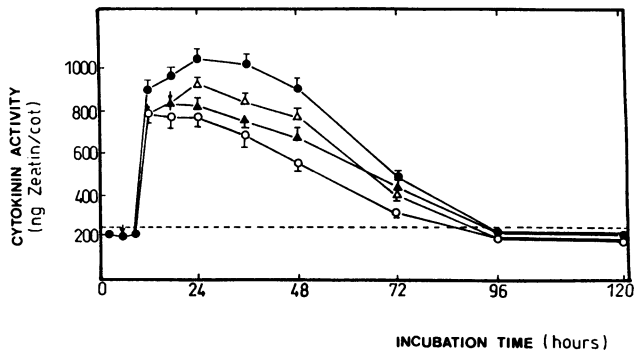

Figure 1. Artificial reproduction of the levels of total cytokinins in excised cotyledons of chick-pea seeds. Control: endogenous cytokinins in normal seeds (●); injection at 6 h of incubation of the cytokinins extracted from cotyledons of seeds germinated for 12 h (○); injection at 12 h of incubation of the cytokinins extracted from cotyledons of seeds germinated for 18 h (▲); injection at 18 h of incubation of the cytokinins extracted from cotyledons of seeds germinated for 24 h (△). Dotted line represents the activity of zeatin corresponding to the control with distilled water. Each point shows the average of three replicates; experiments were repeated three times. Vertical bars indicate SE.

In all cases, the level was slightly lower as a result of a slight retention of the substances in the agar.

In chick-pea seeds, the transport of cytokinins from the axis to the cotyledon takes place between 6 and 24 h of germination (16). This is why no injection of cytokinins is required after 24 h. Ater this time, variations in the levels of cytokinins in excised cotyledons were similar to those found in cotyledons of seedlings (16).

Reproducing the Endogenous Levels of ZR, Z, and Their Respective Glucosides

Reproducing the levels of ZROG in excised cotyledons was done by injection of the corresponding substance at 6, 12, and 18 h of incubation as shown in Figure 2A. A plot profile very similar to that found under germination conditions was found. After 36 h of incubation a small amount of ZR appeared; this increased and showed a maximum level at 48 and 72 h of incubation after which it disappeared at 96 h.

By injection of ZOG at 6, 12, and 18 h (Fig. 2B), it was possible to reproduce the levels of this cytokinin obtained under germination conditions. As may be seen in the figure, after 36 h of incubation a small amount of Z appeared. This increased until 48 h and disappeared at 96 h.

To reproduce the levels of Z (Fig. 2C) it was necessary to inject it at 6 h of incubation; interestingly, at 36 h of incubation there was a certain amount of the dihydro-form (16).

Figure 2D shows the results obtained by injecting ZR after 6, 12, and 18 h of incubation. The corresponding dihydroderivative appeared at 36 h.

The appearance of ZR and Z after 36 h of incubation, coincident with the decrease in ZROG and ZOG shows the existence of a transformation carried out by a β-glucosidase activity in the cotyledons (15) and confirms the results of Smith and Van Staden (23) in maize seeds. The transformation of Z and ZR into their dihydro forms shows that the enzymes necessary for the transformation of the cytokinins into their corresponding dihydro derivatives are present and active in the cotyledons.

Influence of Total Endogenous Cytokinins on Reserve Mobilization

The effect of cytokinins on lipid degradation is shown in Figure 3. When the seeds were germinated there was a rapid decline in the lipid content which was completely prevented by detaching the cotyledons. Addition of total cytokinins partially arrested the decline in lipids. Similarly cytokinins had a profound effect on preventing the loss of total carbohydrates and preventing the buildup of soluble carbohydrates (Fig. 4, A and B) as well as the increase in α-amylase (Fig. 4C).

With processes related to protein metabolism, the results were similar to those obtained for carbohydrates. This holds for protein hydrolysis (Fig. 5A) and protease activity, measured as aminoacid release (Fig. 5B) as well as for peptide release (Fig. 5C).

The results obtained from the studies on reserve mobilization suggest, in agreement with the proposals of Penner and Ashton (21) and Sze and Ashton (25) that the embryonic axis

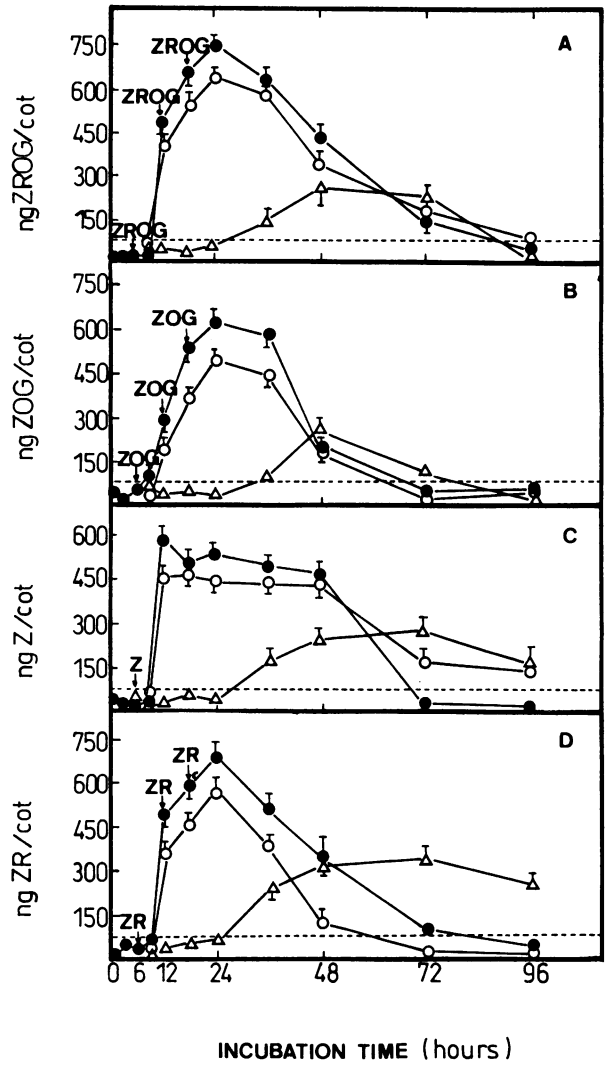

Figure 2. Artificial reproduction of endogenous levels of glucosyl zeatin riboside, glucosyl zeatin, zeatin and zeatin riboside in excised cotyledons of chick-pea seeds. A, Control: endogenous ZROG in normal seeds (●); injection at 6, 12, and 18 h of incubation of the ZROG extracted from cotyledons of seeds germinated for 12, 18, and 24 h respectively (○); ZR appearing as a result of the hydrolysis of ZROG (△). B, Control: endogenous ZOG in normal seeds (●); injection at 6, 12, and 18 h of incubation of the ZOG extracted from cotyledons of seeds germinated for 12, 18, and 24 h respectively (○); Z appearing as a result of the hydrolysis of ZOG (△). C, Control: endogenous Z in normal seeds (●); injection at 6 h of incubation of the Z extracted from cotyledons of seeds germinated for 12 h (○); dihydrozeatin (△). D, Control: Endogenous ZR in normal seeds (●); injection at 6, 12, and 24 h of incubation of the ZR extracted from cotyledons of seeds germinated for 12, 18, and 24 h, respectively (○); dihydrozeatin riboside (△). Dotted line represents the activity of zeatin corresponding to the control with distilled water. Each point shows the average of three replicates; experiments were repeated three times. Vertical bars indicate SE.

controls the mobilization of reserve products of the cotyledons by the production of a hormonal stimulus. In the case of chick-pea seeds, the stimulus seems to implicate the cytokinins, in agreement with the results of exogenous administra-

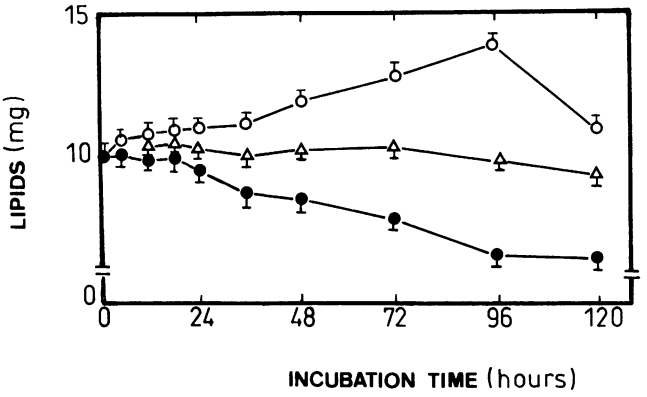

Figure 3. Variation in lipids/cotyledon of *Cicer arietinum* L. seed. Normal seed (●); excised cotyledons (○); excised cotyledons in which the endogenous levels of total cytokinins were artificially reproduced (△). Vertical bars indicate SE.

tion of cytokinins in other dicots seeds (1, 9, 10, 17, 20). The cytokinins seems to be able to replace the influence of the embryonic axis on the mobilization of polysaccharide reserves (Fig. 4, A, B, and C) (27) and on the mobilization of protein reserves (Fig. 5, A, B, and C) (1, 17, 29). However, lipid metabolism (Fig. 3) does not seem to be affected by cytokinins and other substances such as gibberellins could be involved (14).

Influence of ZR, Z, and Their Corresponding Glucosides on Reserve Mobilization in Cotyledons of *Cicer arietinum* L.

Having observed that by injection of the different cytokinins extracted from chick-pea cotyledons it was possible to obtain a fairly accurate reproduction of normal endogenous conditions, the material was collected after treatment for use in the study of the mobilization nutrients.

Neither ZROG nor ZOG was effective in affecting the processes involved in reserve mobilization, *i.e.* changes in carbohydrates, proteins, lipids, amylase and protease activities (data not shown). This result implies that the conjugated forms do not affect reserve mobilization. They may only do so after release of the corresponding free forms (Fig. 2, A and B) (19, 28).

Both ZR and Z affect carbohydrate metabolism. With respect to the variation in total carbohydrates (Fig. 4A) ZR seems to have a more pronounced effect than Z since at 120 h of incubation, for example the recovery of normal values is approximately 60% after application of ZR and 40% after the application of Z. Regarding the variation in soluble carbohydrates (Fig. 4B), the effect of Z and ZR is very similar; however, the studies concerning amylase activity (Fig. 4C) again confirm a somewhat greater recovery of normal levels with ZR than with Z. For ZR a recovery was obtained at 96 h of incubation (the moment which amylase activity is at its maximum) of approximately 82%. For Z, recovery at the moment of maximum activity was only 30% compared with the values obtained in seeds germinated under normal conditions. The promoting effect of the cytokinins on carbohydrate hydrolysis has already been reported for bean cotyledons

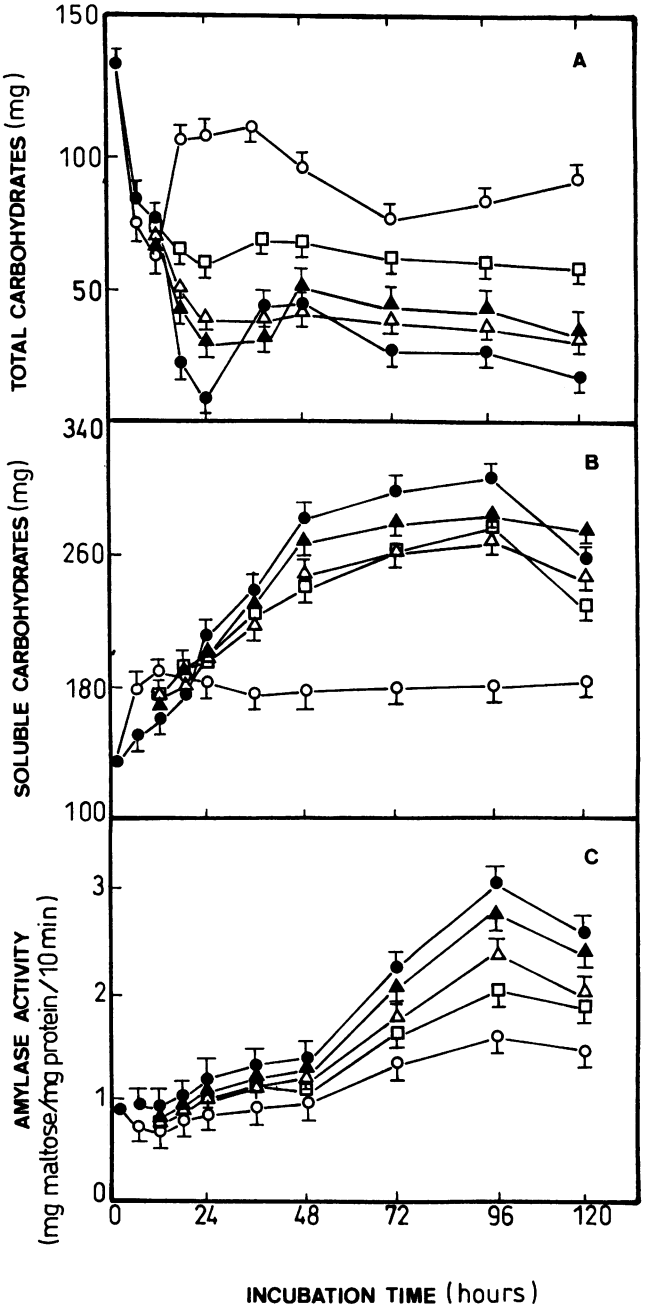

Figure 4. Variation in: A, total carbohydrates/cotyledon; B, soluble carbohydrates/cotyledon; and C, amylase activity/cotyledon of *Cicer arietinum* L. seed. Normal seed (●); excised cotyledons (○); excised cotyledons in which the endogenous levels of total cytokinins (▲); ZR (△) and Z (□) were artificially reproduced. Vertical bars indicate SE.

(9). However, although several authors (14) have proposed that Z is more effective than its riboside in our case, with respect to carbohydrate metabolism, ZR is more effective.

Contrary to what occurs with carbohydrates, Z has much more pronounced effect than ZR on protein metabolism (Fig. 5A). Under treatment with Z in excised cotyledons a recovery of approximately 58% is obtained at 120 h of incubation, whereas with ZR no kind of effect is apparent.

Both Z and ZR affect protease activity measured as the

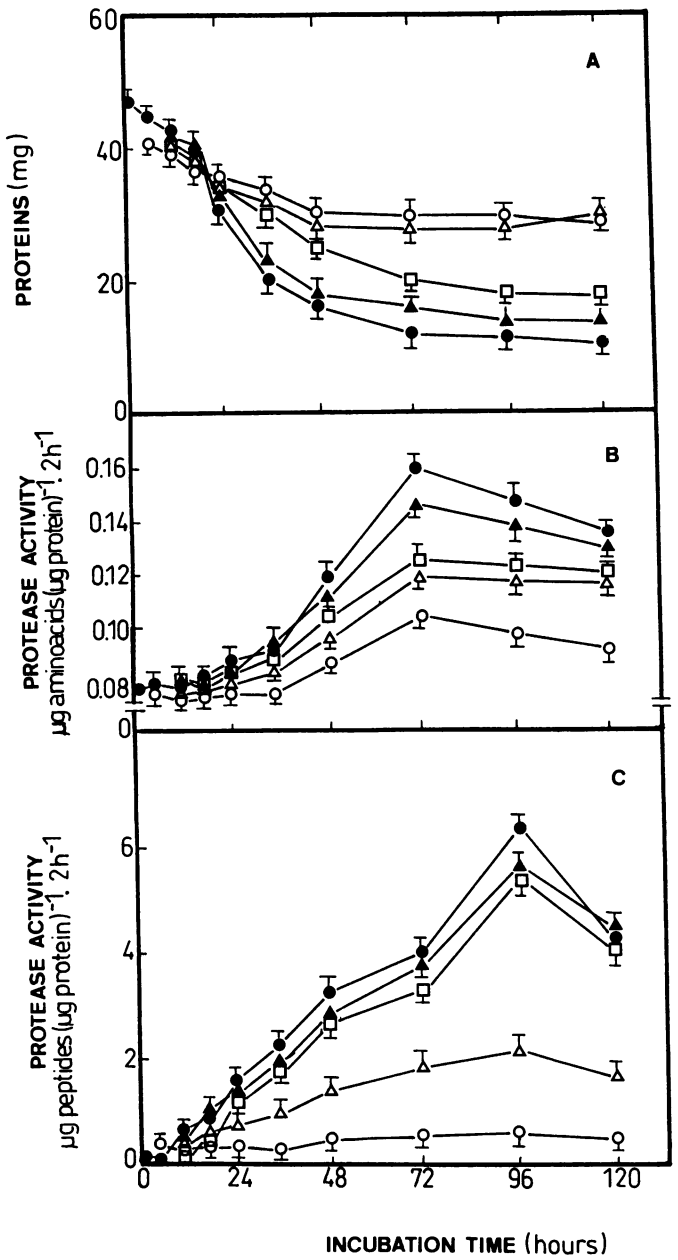

Figure 5. Variation in: A, proteins/cotyledon; B, protease activity (measured as release of aminoacids)/cotyledon; and C, protease activity (measured as release of peptides)/cotyledon of *Cicer arietinum* L. seed. Normal seed (●); excised cotyledons (○); excised cotyledons in which the endogenous levels of total cytokinins (▲); ZR (△) and Z (□) were artificially reproduced. Vertical bars indicate SE.

release of aminoacids (Fig. 5B) and peptides (Fig. 5C) and, especially in the latter case, Z is more effective than ZR. In the case of ZR, recovery is only approximately 25% for the activity measured as amino acid release (Fig. 5B) and 27% when activity is measured as peptide release (Fig. 5C). However, after treatment of the excised cotyledons with Z, recovery of the maximum enzymatic activity measured as peptide release (Fig. 5C) is approximately 82%, whereas the recovery of protease activity measured as amino acid release is only 37% (Fig. 5B). Bearing in mind that the literature mainly includes reports that cytokinins exert a strong influence on protein hydrolysis in excised cotyledons (1, 21) it appears that there is a lack of effect on this process in the particular case of ZR. Thus, regarding these two substances (ZR and Z), two outstanding facts seems to be that in the processes on nutrient mobilization in cotyledons, ZR is more effective on glucosidic metabolism whereas Z has its main effect on protein metabolism. Neither Z nor ZR have any effect on lipid metabolism (data not shown).

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