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Biochemical Changes in Seeds of *Cicer arietinum*, L. During Germination

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M. C. RODRIGUEZ-BUJAN, I. BATALLAN, M. T. BERMUDEZ, R. SANCHEZ-TAMES and G. NICOLAS. *Biochemical Changes in Seeds of* Cicer arietinum, *L. During Germination.* Rev. esp. Fisiol., 30, 21-26. 1974.

The changes of various chemical components, carbohydrates, proteins, lipids, nucleic acids, soluble sugars, total nitrogen, ninhydrin positive materials and phenols in the cotyledons and embryo of *Cicer arietium*, L. seeds have been determined over a 3-day germination period. Extensive loss of carbohydrates and proteins in the cotyledons concomitant with an increase of these major components in the embryo, and soluble sugars and ninhydrin positive materials both in embryo and cotyledons ocurred.

Lipids, DNA and total nitrogen remained relatively constant throughout the experimental period. Increase in RNA during the first 12 h. is followed by a fall, while embryo RNA increased. Phenols also experimented significative changes.

Much information about the metabolic changes in the earliest stages of seed germination has been accumulated in recent years (1, 4, 9, 15, 16). We can consider the metabolism of germinating seeds in two ways: in the sense of degrading reserve materials, and in the sense of producing machinery for protein synthesis and biogenesis of various organelles needded for catabolic and anabolic activities of the new plant.

In this paper the metabolic changes of carbohydrates, proteins, lipids, nucleic acids, soluble sugars, total nitrogen, ninhydrin positive materials and phenols during the first 72 h of germination of *Cicer arietinum*, L., are described.

Materials and Methods

Seeds of *C. arietinum* were germinated in the dark at 25° C on moist filter paper in Petri dishes from 0 to 72 h oven dried at 80° C until constant weight and reduced to a fine powder in a coffee mill. 10 g of this powder was used for the extraction and determination of lipids and nucleic acids. Total lipids were obtained with a choroform: methanol solution (3:1 v/v) by three extractions with 30 ml solvent each time for 1 h at room temperature. The pooled extracts were poured into a preweighed capsule, the solvent was evaporated and total lipid content was determined by the difference between final and

initial weights of the capsule. The residue after lipids extraction was treated with 30 ml of 0.5 N KOH during 1 h at 37° C in order to hydrolyse RNA. Neutralization of the reaction mixture with 4 N HCl and acidification with 5 ml of 50 % TCA was done to precipitate any DNA solubilized by the alkali. The acid reaction mixture was incubated at 0°-4° C during 1 h to ensure DNA precipitation. After centrifugation, the residue was washed with 5 % TCA for complete removal of the hydrolysed RNA. RNA was determined with the orcinol test (17) using as standard similarly treated yeast RNA. The residue from RNA extraction was incubated with 30 ml 5 % TCA at 90° C for 3 h. After vacuum filtration the residue was washed three times with distilled water and filtered, and DNA was estimated by the diprenylamine test (17) using similarly treated salmon sperm DNA as standard.

For the analysis of ninhydrin positive materials and proteins, 30 seeds were powdered and dropped into 100 ml of boiling ethanol and extracted for 5 min. After filtration the residue was extracted with 100 ml of boiling 70 % ethanol for 5 min filtered and extracted again with 100 ml of distilled water. The three extracts were mixed and distilled under vacuum to eliminate ethanol and assayed for ninhydrin positive materials by the method of MOORE and STEIN (14). The solid residue was dried at 60° C for 16 h. 50 mg were suspended in 100 ml of 0.5 N NaOH and incubated at 37° C for 4 h. The supernatant recovered after centrifugation was used for protein determination by LOWRY'S method (13).

After lipid extraction the powder of 30 seeds was treated with 30 ml of 80 % ethanol during 45 min. at room temperature, filtered under vacuum, and reextracted again with an equal volume. The ethanolic extracts were combined, concentrated under vacuum and soluble sugars were estimated by the anthrone test (7) using D-glucose as standard. Total carbohydrates were estimated also by the anthrone test (7) after solubilization of 10 mg of powder in 20 ml of 1 N HCl in a boiling bath for 15 min.

For phenol determination 2 g of powder were extracted four times with 5 ml of 80 % methanol for 2 days. The methanolic extracts were combined, reduced to the aqueous phase in vacuo and partitioned against ether at *p*H 3. The upper phase contains free phenols. To the aqueous layer, HCl was added until a 2 N concentration was achieved, then refluxed for 30 min. and partitioned against ether. The upper phase contains phenols who were as glycosides in the seeds. To 10 ml of the water left after extraction, 20 ml of 1 N $Ba(OH)_2$ were added, refluxed for 90 min and partitioned against ether at *p*H 3. The upper layer contains benzoic and cinnamic acid which were forming esters in the seeds. Each fraction was evaporated to dryness with air, the residue was redisolved in 1 ml of methanol and made up to 25 ml with distilled water. A 7 ml aliquot was taken from each fraction, and phenols evaluated following the method described by SWAIN and HILLIS (18).

Total nitrogen was determined on 50 mg of dried powder by a micro-Kjeldahl method similar to that described by GUARDIOLA and SUTCLIFFE (8). Water content and dry weight of seeds was estimated on samples of 20 seeds from the difference in weight inmediatly after sampling and after drying in an oven at 80° C until constant weight.

All the results are the average of at least three separate experiments with three replicates each one.

Results

Changes in water content and dry weight in seeds of *C. arietinum* are presented in figure 1. Water uptake was maximal during the first 6 h of germination, in this time the percentage of water per seed raised from 9 % in the resting seed to 60 % at

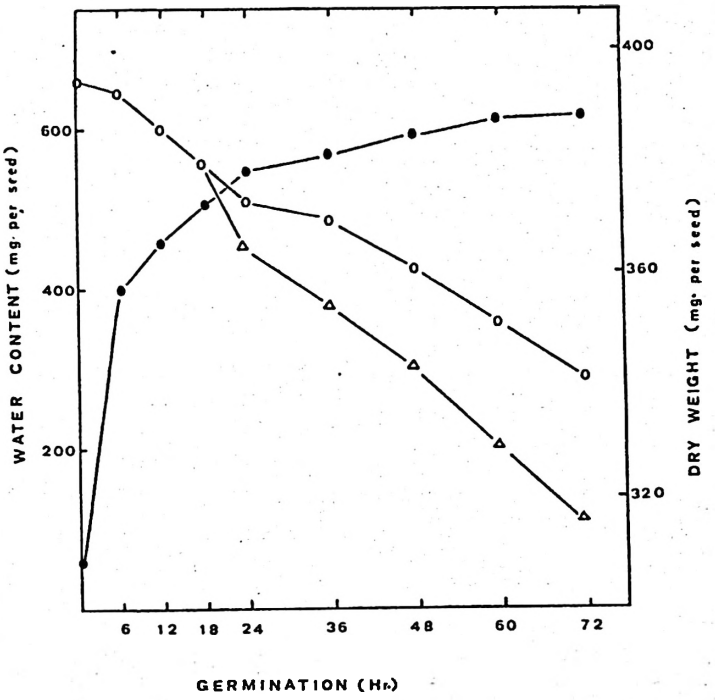

Fig. 1. *Changes in water content (●) and in dry weight in seeds of* C. arietinum: *complete seed (two cotyledons + embryo + testa) (○); minus embryo (△).*

6 h. Water content reached a plateau after about 24 h, thereafter it increased very slowly during the next two days. The loss of dry weight at the end of the germination time was 21 % in cotyledons and 15 % in the complete seed.

In table I, changes in lipids, carbohydrates and soluble sugars are shown. The lipid reserve of cotyledons in resting seeds represents 6.5 % of the total dry weight. This amount experiments only small chages during germination. The carbohydrates content which constitute 69 % of the resting seed decreased progressively during germination. At 72 h the amount of carbohydrates was depleted by 26 % in cotyledons and by 16 % in complete seeds. In contrast, the content in the embryo started to increase from 18 h until 72 h. During germination there was a slight rise in the soluble sugars content in the first 6 h followed by a fall that reached the original level at 18 h, thereafter a new and sharp increase took place in the seed.

Changes in nucleic acids are presented

Table I. *Changes in lipids, carbohydrates, and soluble sugars during germination of* C. arietinum *seeds (mg/seed).*

Complete seed (two cotyledons + embryo + testa): A. Complete seed minus embryo: B. The amount of materials in the growing embryo was deduced by the difference between treatment A and B.

Germination (h)	Lipids		Carbohydrates		Soluble sugars	
	A	B	A	B	A	B
0	25.61	—	270	—	8.86	—
6	25.08	—	257	—	11.90	—
12	24.31	—	249	—	11.43	—
18	22.47	22.50	242	239	7.93	7.93
24	22.16	22.06	236	224	9.33	7.93
36	23.68	23.39	230	214	10.73	8.40
48	23.82	22.88	218	202	12.36	8.88
60	23.52	22.23	225	201	13.91	9.41
72	23.71	22.10	228	201	15.80	10.72

in table II RNA increases during the first 12 h of germination, this rise is followed by a pronounced fall. DNA content remained almos unchanged during the three days. RNA and DNA represented 3 % and 0.21 % respectively of the total dry weight of the resting seed.

In figure 2 it is shown that acid-labile and alkali-labile phenolics follow the same pattern of variation, being the amount of the last about 80 % lower than that of acid-labile phenolics. Free phenolics follow a different pattern, in the first 12 h there is a rapid increase falling down to their lowest value at 18 h and increasing sharply at values about 40 % those of acid-labile phenolics.

Table II. *Changes in RNA and DNA during germination of* C. arietinum *seeds.* Complete seed (two cotyledons + embryo + testa): A. Complete seed minus embryo: B. The amount of materials in the growing embryo was deduced by the difference between treatment A and B.

Germination (h)	RNA (mg/seed) A	RNA (mg/seed) B	DNA (mg/seed) A	DNA (mg/seed) B
0	11.97	—	0.83	—
6	12.70	—	0.90	—
12	13.16	—	1.04	—
18	12.34	10.45	0.99	0.95
24	12.22	9.50	1.05	0.93
36	11.54	9.08	1.11	0.95
48	11.40	8.69	1.12	1.02
60	9.08	8.13	1.05	0.85
72	9.91	8.08	0.99	0.85

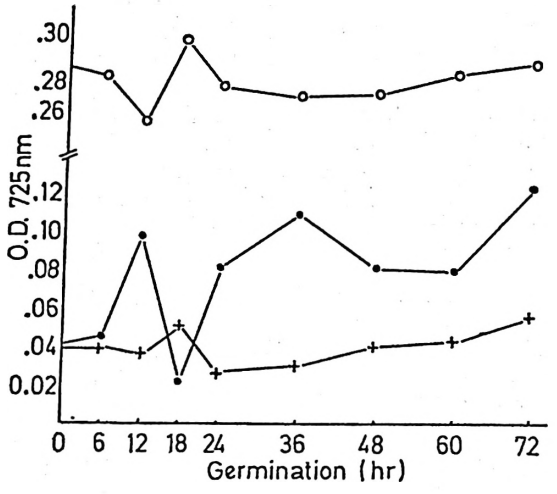

Fig. 2. *Relative amounts of free (●) acid-labile (○) and alkali-labile (x) phenolic compounds in complete seeds of* Cicer arietinum *during germination.*

Table III. *Changes in proteins, total nitrogen and ninhydrin positive materials during germination of* C. arietinum *seeds (mg/seed).* Complete seed (two cotyledons + embryo + testa): A. Complete seed minus embryo: B. The amount of materials in the growing embryo was deduced by the difference between treatment A and B.

Germination (h)	Proteins A	Proteins B	Total nitrogen A	Total nitrogen B	Ninhydrin materials positive A	Ninhydrin materials positive B
0	66.59	—	12.33	—	0.615	—
6	65.46	—	12.35	—	0.803	—
12	63.30	—	12.23	—	0.855	—
18	64.49	62.70	12.46	12.43	0.925	0.927
24	60.21	57.50	12.01	11.76	1.091	1.090
36	57.72	54.24	11.91	14.43	1.720	1.310
48	55.95	52.45	12.49	11.58	2.060	1.620
60	55.62	51.30	11.97	11.05	2.483	2.117
72	51.98	47.85	11.63	9.69	3.071	2.801

The protein reserves of this legume represented about 17 % of the total dry weight. With the onset of germination the amount of proteins (table III) decreased progresively, and at the end of the 72 h the reserves had been depleted by 22 % in the complete seed and by 29 % in cotyledons. Concomitant with this decrease in proteins there was an increase in proteins in the embryo, and an increase in ninhydrin positive materials both in the embryo and cotyledons. The total nitrogen content remained almost unchanged, showing only a small decrease at the end of the germination period.

Discussion

The results here reported are in good agreement with those reported by other

investigators. KOLLÖFEL (11), LARSON (12) and GUARDIOLA and SUTCLIFFE (8) found that water uptake during imbibition of peas seeds was completed after about 24 h. Loss of dry weight is a general phenomenon found during germination of seeds and must be adscribed to respiration since in this proccess carbon is lost as carbon dioxide. Similar results had been found by CHING (6) in pine seeds and by BAIN and MERCER (2) in peas. Since carbohydrates are the main reserve of this legume it is not surprising the drastic changes experimented by this material. From table I we can see that carbohydrates are transformed into soluble sugars and translocated to the embryo where they are used for the synthesis of new polysacharides, probably for the cell walls. We must emphasize that the amount of carbohydrates in the growing embryo at 72 h is about tenfold that at 18 h. Similar results had been found by JULIANO and VARNER (10) in rice.

It has been observed that anaerobic respiration takes place in the first twenty hours of germination (unpublished data). The phenomenon can be due to: *a*) non funtioning mitochondria, *b*) envelope impermeability to oxygen and *c*) lack of oxygen in the embryo, being trapped by free phenolics. If the last mechanism is operative, as COME (5) demonstrated in apple seeds, when the amount of free phenolics decreases, respiration becomes aerobic and at this point the radicule protrudes, oxygen reaches freely the embryo and the synthesis of phenolic compounds starts, increasing the amount of free phenolics.

The decrease in proteins in the cotyledons with a parallel accumulation of proteins in the embryo and the rise of ninhydrin positive materials resembles the situation in peas (3). We must point out the fact that the accumulation of protein and total nitrogen in the embryo started at 18 h but we could not detect accumulation of ninhydrin positive materials until 36 h (table III). This seems to indicate that amino acids, as soon as they reach the embryo are used immediatly in the synthesis of proteins.

Because of the known close relation between nucleic acid synthesis and protein synthesis it would be expected that the rapid protein hydrolysis and synthesis taking place in the cotyledons and in the growing embryo, respectively, would be correlated with a marked synthesis of RNA, as we have found that occurs in *C. arietinum* seeds. During the first 12 h of germination we can detect a progressive increase in RNA followed by a rapid degradation. The resultant nucleotides will be translocated to the embryo and used in RNA synthesis; in fact it is possible to detect accumulation of RNA in the embryo at 18 h of germination. These changes in RNA are in general agreement with the findings of BEEVERS and GUERNSEY (3). The general trend of compositional changes in *C. arietinum* follows that of a typical starchy seed.

Resumen

Se han estudiado los cambios químicos que tienen lugar en los cotiledones y embrión de las semillas de *Cicer arietinum* durante los tres primeros días de germinación. Se han determinado carbohidratos, proteínas, lípidos, ácidos nucleicos, azúcares solubles, nitrógeno total, materiales positivos a la ninhidrina y fenoles. Carbohidratos y proteínas muestran una considerable disminución en los cotiledones, aumentando paralelamente la cantidad de azúcares solubles y material positivo a la ninhidrina. En el embrión, por el contrario, se observa un aumento constante en proteínas y carbohidratos.

Los lípidos, nitrógeno total y DNA permanecen sin variación apreciable durante el período experimental. El RNA aumenta durante las primeras 12 h de germinación para después disminuir bruscamente. Los fenoles también experimentan cambios significativos.

Bibliografía

1. ABDUL-BAKI, A. A.: *Plant Physiol.*, 44, 733-738, 1969.

2. BAIN, J. M., and MERCER, F. V.: *Aust. J. Biol. Sci.*, **19**, 69, 1966.
3. BEEVERS, L., and GUERNSEY, F. S.: *Plant Physiol.*, **41**, 1455, 1966.
4. BEEVERS, L., and SPLITTSTOESSER, W. E.: *J. Exp. Bot.*, **19**, 698, 1968.
5. COME, D.: *Ann. Sci. Nat. Bot.*, **8**, 371, 1967.
6. CHING, T. M.: *Plant Physiol.*, **45**, 475, 1970.
7. CHUNG, C. W., and NIKERSON, W. J.: *J. Biol. Chem.*, **208**, 395, 1954.
8. GUARDIOLA, J. L., and SUTCLIFFE, J. F.: *Ann. Bot.*, **35**, 791, 1971.
9. INGLE, J., BEEVERS, L., and HAGEMAN, W.: *Plant Physiol.*, **39**, 735, 1964.
10. JULIANO, B. C., and VARNER, J. E.: *Plant Physiol.*, **44**, 886, 1969.
11. KOLLÖFEL, C.: *Acta Bot. neerl.*, **16**, 111, 1967.
12. LARSON, L. A.: *Plant Physiol.*, **43**, 255, 1968.
13. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J.: *J. Biol. Chem.*, **193**, 265, 1951.
14. MOORE, S., and STEIN, W. H.: *J. Biol. Chem.*, **211**, 907, 1954.
15. MOROHASHI, Y., and SHIMOKORIYAMA, M.: *J. Exp. Bot.*, **23**, 45, 1972.
16. PALMIANO, E. P., and JULIANO, B. O.: *Plant Physiol.*, **49**, 751, 1972.
17. SCHNEIDER, W. C.: «Methods in Enzymology». Vol. 3 (S. P. Colowick and N. O. Kaplan, eds.). Academic Press, pp. 680, New York, 1957.
18. SWAIN, T., and HILLIS, W. E.: *J. Sci. Food Agric.*, **10**, 63, 1959.