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CHANGES IN PHENOLIC COMPOUNDS DURING THE GERMINATION OF SEEDS OF *Cicer arietinum* L.

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RESUMEN

Se estudia el contenido de diferentes tipos de derivados fenólicos a lo largo de la germinación de semillas de *Cicer arietinum* L. La presencia del embrión influye en el contenido de fenoles libres, que al final del experimento alcanza valores más altos en semillas enteras que en semillas desprovistas de embrión. Se aislaron varios compuestos fenólicos de los cuales se dan datos cromatográficos y reacciones coloreadas y se identificaron: ácido p-hidroxi benzóico, ácido vanillico, ácido siríngico, ácido p-cumárico, ácido ferúlico, ácido gentísico, p-hidroxi acetofenona y garbanzol.

ABSTRACT

The content of differently linked phenol compounds during germination of seeds of *Cicer arietinum* L. has been studied. The presence of the embryo influences the content of free phenols which at the end is greater than in seeds without embryo. Several compounds have been isolated and p-hydroxybenzoic acid, vanillic acid, syringic acid, p-coumaric acid, ferulic acid, gentisic acid, p-hydroxy acetophenone and garbanzol identified. For the unidentified compounds chromatographic data and colour reactions are given.

INTRODUCTION

Under the heading phenols, several structurally different compounds are included; these compounds are widely distributed in the seed components (VAN OVERBEEK, 1966). Although they were considered as germination inhibitors they can promete germination at different concentrations (CÔME, 1970), sometimes both effects can be shown in the same solution (EVENARI, 1949). It is clear that phenols play an important role in barley seed germination and it is suggested that amino acid and protein synthesis may be affected by phenols (VAN SUMERE *et al*, 1972). As part of a study on the germination of seeds of *Cicer arietinum* L.

changes in concentration of phenol compounds and the isolation and identification of several of such compounds is reported.

MATERIALS AND METHODS

Plant material

Intact seeds of *Cicer arietinum* L. or only cotyledons and testa were sterilized by immersion for 1 min in 0.1 % $HgCl_2$ and afterwards washed for 3 h in sterile distilled water. Germination was carried out in Petri dishes lined with wet towel paper, in a dark room with 80 % relative humidity and 25 ± 1°C from 0 h to 72 h. The seeds used for quantitative evaluation of phenols were dried at 80°C to constant weight and milled to a fine powder.

Extraction and purification

All solvents were redistilled before use and the ethyl ether freed of peroxides. Weighted quantities of seeds were suspended in 80 % methanol for 24 h at room temperature. After filtration the residue was reextracted three times with 80 % methanol for 12 h at room temperature. The filtrates were combined, the methanol was removed under vacuum at 34°C, then the aqueous residue was acidified to pH 3,0 with 2N HCl, the free phenols were extracted by partition four times against equal volumes of ethyl ether. The combined ether layers were evaporated to dryness, this was called direct fraction and contained free phenols and those forming salt type combinations. The aqueous residue was hydrolyzed with 2N HCl and heated in reflux for 30 min. The hydrolysate was extracted with ethyl ether as before. This was called the acid fraction and contains phenolic compounds extracted as glycosides.

The aqueous residue left after hydrolysis was adjusted to pH 8 with $Ba(OH)_2$ and refluxed for 2 h, followed by adjustment to pH 3 by 2N HCl and partitioned four times against ethyl ether. The combined ether layers were evaporated to dryness. This was called the alkaline fraction, and contained compounds extracted as esters.

The plant material left after methanol extraction was refluxed with 2N OHNa for 4 h, acidifed to pH 3 and partitioned four times with ether. The ether fractions were combined and evaporated as before. This was the residual fraction.

Each dry extract was redissolved in 10 ml 80 % MeOH.

Analytical methods: Total phenols were measured by Folin-Denis as described by SWAIN & HILLS (1959) using extract aliquots of 7 ml. Absorbance at 725 nm was compared with a gentisic acid calibration curve, and expresed as µg of this compound.

The isolation and identification of the phenol compounds was made by paper

chromatography on Whatman N.º 1 & 3 MM and TLC on cellulose and silica gel. The solvents used for developing the chromatograms were: S_1, isopropanol ammonia-water (10:1:1 v/v); S_2, benzene-acetic acid-water (6:7:3 v/v); S_3, chloroform-methanol – 4 % formic acid (10:1:1 v/v); S_4, 2 % acetic acid.

It was necessary to run several chromatograms in order to achieve a good separation of the compounds. The elution of the isolated products on paper or TLC was done with methanol (ESHDAT and MIRELMAN, 1972).

To identify compounds the following methods were used. The chromatograms were examined in UV –366 and 254 nm– before and after application of ammonia vapour. They were also developed by spraying with one of the following solutions: diazotized p-nitroanyline (C_1), 2,6 -dibromoquinonechlorimide (C_2), diazotized sulphanilic acid (C_3) or phosphomolibdic acid (C_4).

The absorption spectra were run from 250 to 340 nm in methanol, in 5 % KOH in methanol or 5 % $AlCl_3$ in methanol. The distribution of phenols during germination was determined by bidimensional chromatography of equivalent extracts of seeds from the different periods assayed and the solvents used were S_1 for the first run and S_2 for the second run.

RESULTS AND DISCUSSION

In a previous work (RODRÍGUEZ-BUJÁN *et al.*, 1974) we have evaluated some types of differently linked phenol compounds during germination. Now we compare the phenol content –different fractions– during germination between intact seeds (Fig. 1) and seeds without embryo (Fig. 2).

From these data, it is shown that the content of free phenols changes along germination, increasing in the whole seed while in embryoless seed there is a continuous decrease in phenol content.

The alkaline fraction-containing the ester forming phenols–, keeps a fairly constant level during the 72 h period. There is no significant difference between intact and embryoless seeds.

The acid fraction, that is glycoside forming phenols, keeps fairly constant levels during germination in the intact seeds, while in embryoless seeds there is a greater variation. After 72 h the content has decreased to half that at 6 h.

In conclusion it can be said that, at 72 h there are twice as many physiologically important phenols – free + glycosides + esters – in intact compared to embryoless seeds.

Residual phenols, those liberated after alkaline hydrolysis, and probably less related to the germination process, increase their content in both cases.

It is interesting to notice, that around 18-24 h of germination has been found to be a very important point (RODRÍGUEZ-BUJÁN *et al.*, 1975, DE LA FUENTE y NICOLÁS, 1974) probably related with the change from anaerobic to aerobic respi-

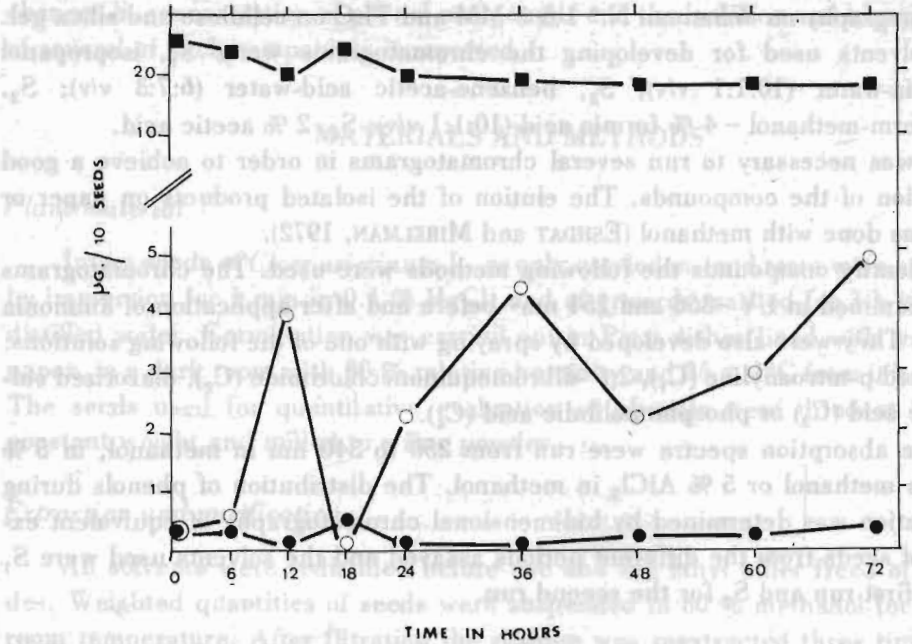

Fig. 1.–Amounts of free (–o–), acid labile (–■–) and alkali labile (–●–) phenols in whole seeds of *C. arietinum* during germination, expressed as g of gentisic acid per ten seeds. Each point is the mean of 3 replications.

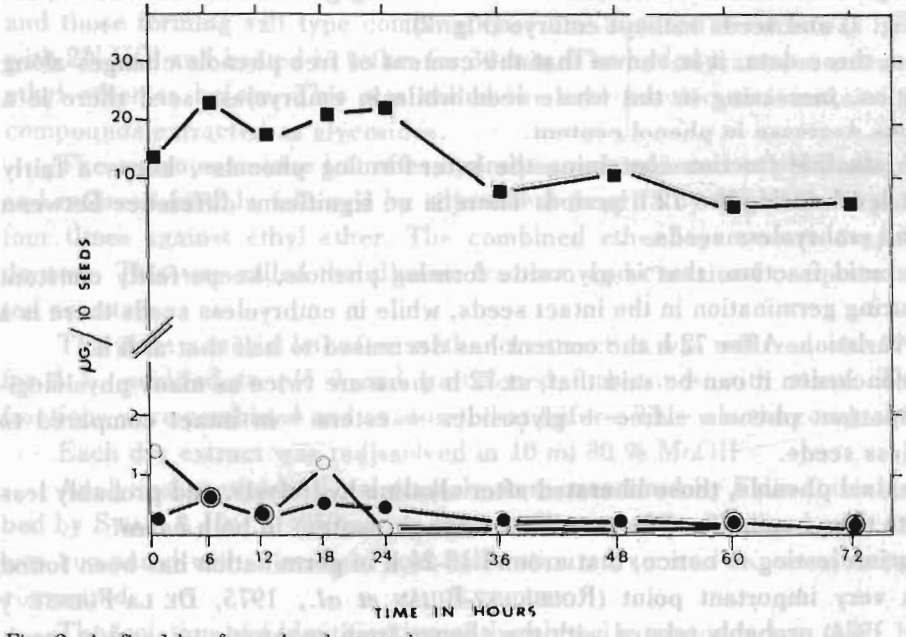

Fig. 2.–As fig. 1 but for embryoless seeds.

ration, due to root protrusion through the envolope and we can see that this is a turning point also for the free phenols, which seem to be mostly related to germination of the seed, probably in the trapping of oxygen (CÔME, 1970). Once the radicle has protruded the phenols are not required to the same extent and the content of free phenols increases as a result.

The identified compounds were: three benzoic acid derivatives (p-hydroxybenzoic acid, vanillic acid and syringic acid), two cinnamic acid derivatives (p-coumaric acid and ferulic acid), gentisic acid, p-hydroxyacetophenone and garbanzol, although the identity of this last compound is based on indirect evidence due to lack of a pure sample. Another eight phenolic compounds have been isolated but not identified (see Table I and II). The semiquantitative analysis

TABLA I

Chromatographic properties of the unidentified compounds (For explanation of symbols see methods)

Compounds	Rf values				Colour reaction					
	S_1	S_2	S_3	S_4	C_1	C_2	C_3	UV_{366}	$UV_{366} + NH_3$	UV_{254}
Unknown 1	0.54	0.70	0.95	0.70	be-o	bl	y-o	–	–	p
Unknown 2	0.70	0.00	0.30	0.50	vt	–	–	bl	–	–
Unknown 3	0.30	0.25	0.36	0.55	vt	–	–	bl	–	bl
Unknown 4	0.12	0.43	–	–	pk	–	–	–	–	p
Unknown 5	0.80	0.48	–	–	–	–	–	bl	–	–
Unknown 6	0.70	0.26	0.80	0.89	–	–	–	–	–	p
Unknown 7	0.60	0.65	0.64	0.11	–	–	–	y	gn	–
Unknown 8	0.35	0.30	0.95	0.05	be-o	bl	y-o	–	–	p

Key to colours.–be: beige; bl: blue; gn: greenish; o: orange; p: purple; pk: pink; vt: violet; y: yellow.

TABLE II

Ultraviolet absorption peaks (in methanol) for the unidentified compounds of *C. arietinum* seeds. (For explanation of symbols see methods)

Compounds	max (nm)		
	MeOH	KOH	$AlCl_3$
Unknown 1	262	274, 329s	270
Unknown 2	281	279	272
Unknown 3	283	284	278
Unknown 4	253	283, 257s	269, 248s
Unknown 5	285	281	282
Unknown 6	280	281	284
Unknown 7	270, 276, 310s	275, 334	268, 277, 310s
Unknown 8	256, 278s	262, 315s	243

of the isolated compounds has been determined during germination as shown in Table III; p-hydroxybenzoic, vanillic and syringic acid were found in all the

TABLE III

Presence (+) or abscence (−) of the isolated compounds during germination

Compounds	Time in hours																											
	0 h			6 h			12 h			18 h			24 h			36 h			48 h			60 h			72 h		
	Dr	Ac	Al	Dr	Ac	Al	Dr	Ac	Al	Dr	Ac	Al	Dr	Ac	Al	Dr	Ac	Al	Dr	Ac	Al	Dr	Ac	Al	Dr	Ac	Al
p-Hydoxybenzoic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vanillic acid	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Syringic acid	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+
Gentisic acid	−	+	+	−	+	+	+	+	−	−	+	+	−	+	+	+	+	+	+	−	+	+	+	−	−	+	+
p-Coumaric acid	+	+	+	+	−	+	+	−	+	+	+	+	+	+	+	+	−	−	+	−	+	+	−	+	−	−	+
Ferulic acid	−	−	+	−	−	+	−	−	−	−	−	+	−	−	+	+	−	+	+	−	+	+	+	+	+	+	+
p-Hydroxy Acetophenone	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Garbanzol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	+
Unknown 1	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	+	−
Unknown 2	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	−	−
Unknown 3	−	+?	−	−	+	+	−	+	+	−	+	+	−	−	+	−	+	+	−	+	+	−	+	−	−	+	−
Unknown 4	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	−
Unknown 5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−
Unknown 6	−	+	+	−	+	+	−	+	+	−	−	+	−	+	−	−	+	+	−	+	+	−	+	−	−	+	−
Unknown 7	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+
Unknown 8	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−

Dr: direct fraction, Ac: acid fraction, Al: alkaline fraction.

fractions studied. These phenolic acids are widely distributed in all plants their presence being related with lignin biosynthesis, although in the early steps of germination it does not seem to be their main role. This could however explain the presence of syringic acid which occurs only after 60 h in the alkaline fraction.

Initially, p-coumaric acid is detected free and in two bound forms, afterwards at 72 h only is detected as ester-linked compound. The behaviour of ferulic acid is different, there is no free or glycoside linked ferulic acid for the first 24 hours, at 36 h there is some free ferulic acid and at 60 h is present as free glycoside or ester linked.

Gentisic acid is not detected in the beginning as a free acid, although later on it shows fluctuations, between 36 and 60 h it constantly appears as free acid.

At 0 h p-hydroxyacetophenone is present as glycoside.

Garbanzol appears as ester forming compound after 36 h of germination.

The isolated compounds must play a role in the regulation of seed germination and early stages of seedling development. The embryo is needed for phenol production or mobilization, as free or glycoside forming compounds are those which experiment greater fluctuations along germination and also greater differences between the content of whole and embryoless seeds.

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