Influence of gibberellic acid on hydrolytic enzymes activity in germinating different-aged lupin seeds

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Abstract

The influence of GA_3 on the germination of different-aged lupin seed and α-amylase, protease and ribonuclease activity in the embryonic axis and cotyledons of these seeds were investigated after 24, 48, 72 and 96 h of germination. GA_3 treatment of older seeds greatly reduced the per cent of abnormal seedlings and increased ribonuclease activity, and, at the beginning of germination, also of amylase, whereas protease activity remained unchanged. Young seeds did not show any definite reaction to GA_3 with the exception of a slight increase in ribonuclease activity.

INTRODUCTION

The scarse investigations on metabolism of ageing seeds indicate that one of the symptoms of their degradation is the lowered hydrolytic enzymes activity during germination (A n d e r s o n, 1968; K u l k a, 1971; Ł u c z y ń s k a, 1973). The cause of this lies probably in the loss by the seeds of the ability to synthesize certain proteins, mainly high molecular weight albumins involved in the structure of enzymatic systems (G r z e s i u k and K u l k a, 1971b; K u l k a, 1971). Another cause of the decrease in hydrolase activity may be hormonal disturbances owing to which the controlling role of the embryo in the mobilization of storage substances of the germinating seeds becomes weaker (F l o r i s, 1970).

In order to, at least partly, elucidate this problem, the influence of exogenous giberellic acid on the course of germination and the activity of some enzymes in different-aged lupin seeds was investigated.

MATERIAL AND METHODS

The experiments were made with two lots of lupin seed (*Lupinus luteus*) variety 'Popularny' received from the Department of Seed Biology and Storage, Institute of Plant Breeding and Acclimatization (IHAR) in Wrocław. The first lot of "old" seeds collected in 1966 had been stored for 6 years in tight containers at about 20°C and air humidity 50%. The second lot of "young" seeds collected in 1970 were stored in identical conditions for 2 years.

1. Germination

The germination energy and germination ability of seeds were checked by the method developed in the Department of Seed Biology and Storage for large-seed *Papilionaceae*. According to this method control seed samples (100 in each sample in 4 replications) were first sterilized in 1% sodium hypochlorite and placed on 4 layers of filter paper moistened with water up to saturation. Then the filter paper with the seeds was rolled up and the rolls were placed in cylinders of 500 ml capacity containing 20 ml of water, which were put in a thermostat adjusted to 20°C.

For the experimental samples the filter paper was saturated with a GA_3 water solution of 200 mg/l concentration instead of water. This concentration was considered as most suitable on the basis of preliminary orientational investigations in which 100, 200 and 300 mg/l solutions were used. To the cylinders 20 ml of GA_3 solution was also added instead of water.

2. Determination of enzyme activity

The activity of α-amylase, protease and ribonuclease was determined in 4 replications in the cotyledons and embryonic axis of germinating seeds after 24, 48, 72 and 96 h of germination. In order to obtain the most reliable results the entire seed sample, i.e. 100 seeds were taken for analysis. The enzymatic extract was prepared by homogenization of the material in acetate buffer of pH 6.0 at 0—2°C for 0.5 h. Then the homogenate was filtered through four layers of cheese-cloth and centrifuged for 10 min at 2000 g. The supernatant was taken for analysis.

α-Amylase activity was determined by the starch-iodine method described by J o n e s and V a r n e r (1967).

The reaction mixture contained the enzymatic extract in a 0.1 to 0.2 ml volume + 1 ml of starch substrate. After 5 min of incubation at 20°C the reaction was stopped by addition of 1 ml of the iodine reagent, 5 ml of water was added and optical density (OD) was read at 620 nm. Within the range of concentrations tested the decrease of optical density was directly proportional to the α-amylase content in the reaction mixture. The results were converted to 1 μg amylase with the use of the conversion factor obtained when incubating a standard starch sample with a purified α-amylase preparation (Fluka AG) according to the formula

$$1 \ \mu g \ a-\text{amylase} = \frac{\varDelta OD \times Tv \times C.F.}{t \times v}$$

where: ΔOD — difference in optical density between the control and the experimental sample

  Tv — volume of enzymatic extract from whole sample investigated;

  C.F. — conversion factor;

  t — incubation time;

  v — volume of supernatant taken for incubation.

The starch substrate was prepared every day from 150 mg of insoluble starch + 600 mg KH_2PO_4 + 200 μM $CaCl_2$ made up with water to 100 ml. The suspension obtained was boiled for 1 min, cooled and centrifuged for 10 min at 3000 g. The clear supernatant was used as test substrate.

The iodine reagent was prepared from 6 g KI + 600 mg I_2 disolved in 100 ml water. To 1 ml of this solution 0.05 N HCl was added up to 100 ml and the mixture was used to stop the reaction.

Protease activity was determined by the modified Anson method described by K u l k a (1971). The reaction mixture containing 1 ml of enzymatic extract + 1 ml of prepared casein + toluene as antiseptic was incubated for 24 h at 36°C. Then proteolysis was stopped by adding 3 ml of 7⁰/₀ trichloroacetic acid. The sample was cooled at —10°C for 15 min and centrifuged at 5000 g. In the supernatant tyrosin content was determined colorimetrically with the use of the Folin-Ciocalteau reagent and of a red filter (670 nm). Protease activity is expressed in μmoles of liberated tyrosin.

Ribonuclease activity was determined by the somewhat modified method of Tuve and Anfinsen (1960). The reaction mixture consisted of 2 ml of 0.2⁰/₀ RNA (yeast) solution in 0.1 M acetate buffer pH 6.0 + 0.5 ml of enzymatic extract. After 2-h incubation at 37°C the reaction

was stopped by adding 0.5 ml 0.75% uranyl acetate in 25% perchloric acid. The sample was cooled and centrifuged at 6000 g. To 0.2 ml of supernatant 4 ml water was added and extinction was measured at 260 nm in a 1 cm layer.

As unit of ribonuclease activity, an amount of enzyme was adopted which, under the prevailing experimental conditions, increased extinction in the supernatant as compared with the control sample by 0.1.

RESULTS AND DISCUSSION

Germination

Observation of the course of germination demonstrated that 6-year-old seeds exhibit not only a reduced germination ability but that a large number (more than 50%) of seedlings are abnormal (Table 1).

Table 1

Influence of GA_3 on germination of different-aged lupin seeds

Year of harvest	Combination	Germinating seeds, %		Abnormal seedlings %		Mean length of seedlings cm							
						after 4 days				after 8 days			
						normal		abnormal		normal		abnormal	
		after 4 days	after 8 days	after 4 days	after 4 days	hypo-cotyl	root	hypo-cotyl	root	hypo-cotyl	root	hypo-cotyl	root
1966	Control	70	72	55	50	1.41	2.96	1.01	1.29	4.12	7.36	2.73	2.49
	GA_3	71	73	32	28	1.37	2.82	1.12	1.23	4.38	7.41	2.89	2.54
1970	Control	94	96	22	19	1.52	3.56	1.23	1.51	4.86	8.32	3.08	3.17
	GA_3	94	96	20	16	1.57	3.48	1.36	1.62	4.95	8.27	3.23	3.05

The most frequent cause of defective germination occurring in lupin may be damage in the course of development and harvesting, the noxious influence of chemical factors and internal changes due to ageing. In the present case poor germination was no doubt caused by the biological decline of viability, since, according to the data of the Department of Seed Biology and Storage, the initial quality of the seeds had been normal.

The abnormality of the seedlings consisted in a considerable shortening of the root and hypocotyl, disproportion in their dimensions, torsion and constriction (Photo 1, a). A detailed morphological classification of the symptoms, which, according to the author are an indirect indice of ageing, is given by S w i r s k a (1964).
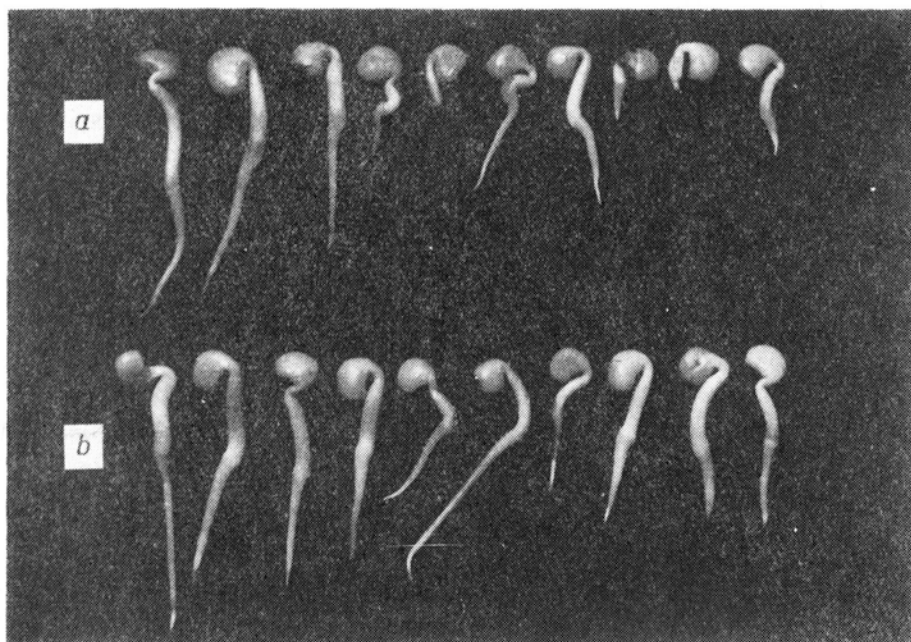
Photo. 1. Seedlings from 6-year-old seeds after 96 h of incubation. a — control; b — with GA$_3$
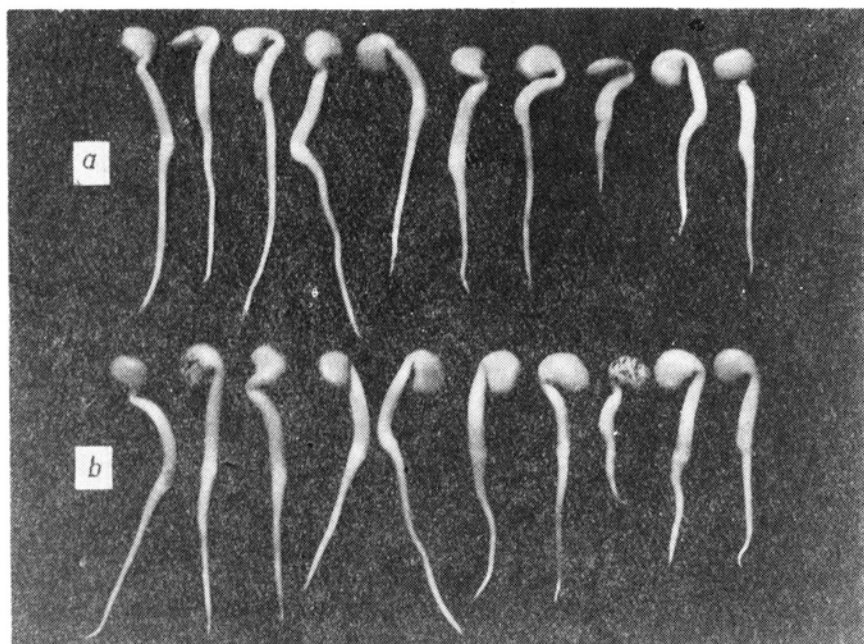

Photo. 2. Seedlings from 2-year-old seeds after 96 h incubation. a — control; b — with GA$_3$
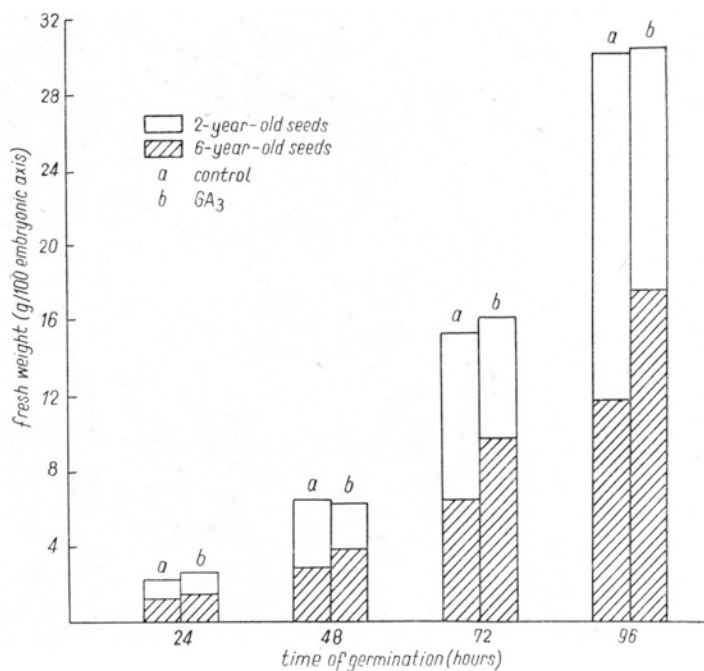
Fig. 1. Influence of GA$_3$ on fresh mass increment of embryonic axis isolated from different-aged lupin seeds
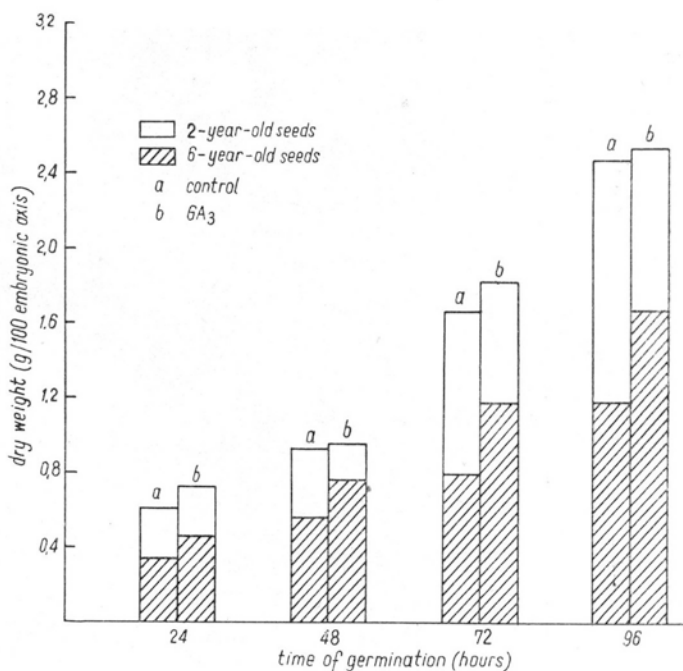

Fig. 2. Influence of GA$_3$ on dry mass increment of embryonic axis isolated from different-aged lupin seeds

Treatment of 6-year seeds with GA₃ solution did not affect their germination ability in general, but greatly decreased the number of abnormal seedlings (Table 1, Photo 1). The shape of seedlings changed, the hypocotyl elongated and there were hardly any torsions. In many seedlings however, the disproportion between the length of the hypocotyl and root remained, so that this decided of their classification. Essential changes in the morphological structure of the seedlings which made possible their classification as normal appeared after 4 days. Prolongation of observations of germination up to 8 days only slightly changed the results.

The marked elongation of the hypocotyls induced by GA₃ cannot be considered as a mean increment in length in the entire seed sample since the differences between the normal and defective seedlings are too wide. As measure of the growth reaction here may be adopted in the first place the increased per cent of normal seedlings (Table 1) and the increment in fresh and dry mass in the whole sample (Figs 1 and 2).

In contrast to old seeds, treatment with GA₃ of 2-year-old ones did not cause major changes in the development of their seedlings (Table 1, Photo 2). It is not excluded that this lack of reaction is connected with the very high level of endogenous gibberellins in lupin, which reaches in some varieties 17 μg/g of fresh weight (cf. P h i n n e y and W e s t, 1960).

It should be stressed that in spite of the distinct influence of GA₃ on the morphological structure of the seedlings of old seeds, their quality was much inferior to that of the younger ones which showed a quicker and better growth.

Enzyme activity

The results listed in tables 2—4 show that degradation of old seeds manifested in abnormal germination only slightly affected the activity of hydrolytic enzymes.

α-Amylase activity in the embryonic axis of old seeds was at the beginning of germination three times less that in the young seeds, but after 96 h it almost reached an equal level. In the cotyledons in both lots of seeds α-amylase activity was equal after 48 h of germination (Table 2).

K u l k a (1971) while investigating amylase activity in oat grains after 7-year storage, exhibiting very poor viability, found that it was many times lower as compared with normal good seed material. On the other hand, in 4-year-old grain with a germination power above 70 per cent the seedlings were shortened, and the activity of amylolytic enzymes was relatively high. Similar data are reported by A n d e r s o n (1968) in investigations on ageing barley grain.

Table 2

α-Amylase activity in germinating lupin seeds of different ages treated with GA_3
(in mg α-amylase/100 seeds)

Year of har-vest	Combina-tion	Germination time							
		24 h		48 h		72 h		96 h	
		axis	cotyle-dons	axis	cotyle-dons	axis	cotyle-dons	axis	cotyle-dons
1966	Control	1.06	4.20	2.30	7.14	3.42	8.10	4.54	11.34
	GA_3	2.84	6.30	3.53	7.45	4.31	8.96	4.82	11.76
1970	Control	3.29	6.16	3.98	7.42	4.59	8.24	5.04	12.14
	GA_3	3.47	6.64	3.81	7.84	4.42	9.10	5.10	11.90

The results reported here seem to point to a certain inhibition of synthesis or release of the above mentioned enzyme in 6-year lupin seeds, which, however, seems to recede in the course of further development of the seedlings.

Protease activity in the axis and cotyledons of old seeds differed but little from that in young ones (Table 3). This supports the results of Łuczyńska (1973) for soybean and of Ching and School-kraft (1968) for clover. The latter authors suggests that the unaltered

Table 3

Protease activity in germinating lupin seeds of different ages treated with GA_3
(in μmoles tyrosin /100 seeds)

Year of har-vest	Combina-tion	Germination time							
		24 h		48 h		72 h		96 h	
		axis	cotyle-dons	axis	cotyle-dons	axis	cotyle-dons	axis	cotyle-dons
1966	Control	23.2	66,0	36,0	126.0	41.6	196.8	49,2	220.4
	GA_3	28.8	72.0	39.2	142.0	44.8	198.0	52.4	218.2
1970	Control	28.0	84.0	40.8	145.0	48.0	220.4	57.2	234.6
	GA_3	30.4	80.0	38.4	148.0	48.0	215.6	59.8	248.4

proteolytic enzymes activity during ageing is connected with the degradation of structural proteins. This would explain the relatively high protease level found even in dead seeds of certain species (Barton, 1961).

Wide differences were noted in ribonuclease activity between old and younger seeds (Table 4). Interpretation of this fact is based on earlier work elucidating the role of ribonucleases in the process of seed germination (cf. Key, 1969; Gołaszewski et al., 1972). Disturbances in the metabolism of particular forms of NA were detected in ageing barley and oat seeds, progressing parallelly with the considerable depression of ribonucleolytic enzymes activity (Grzesiuk, Kulka, 1971a).

Table 4

Ribonuclease activity in germinating lupin seeds of different ages treated with GA₃
(in ribonuclease units R.u.)

Year of harvest	Combination	Germination time							
		24 h		48 h		72 h		96 h	
		axis	cotyledons	axis	cotyledons	axis	cotyledons	axis	cotyledons
1966	Kontrola	81	222	192	308	576	1062	1096	2350
	GA₃	72	206	368	590	1024	1636	1728	3182
1970	Kontrola	80	298	504	770	1152	1882	1928	3338
	GA₃	96	284	688	936	1296	2128	2040	3664

Analysis of the results concerning the influence of GA₃ on enzyme activity shows that the reaction of different-aged seeds in this respect is not identical. In young seeds treated with GA₃ a-amylase activity did not change, whereas in old ones the effect of the hormone was manifest not in the general increase of this activity, but in its acceleration. As seen in table 2, a-amylase activity in the axis of old seeds reached a similar level as in young ones under the influence of GA₃ as early as after 24 h of incubation, whereas in the control seeds this occurred after 96 h.

Proteinase activity in seeds of both lots treated with GA₃ remained without major change (Table 3), both in cotyledons and in the axis.

The hormone proved most effective as regards ribonuclease (Table 4). After 48 h of incubation with GA₃, ribonuclease activity in the particular parts of old seeds increased almost twofold. In young GA₃-treated seeds an increase in RNA-ase activity as compared with the control was also observed, it was not so pronounced, however, as in the case of old seeds.

The stimulating action of GA₃ on synthesis and secretion of some hydrolases in the storage parts of seeds and the intact seeds has been noted many times (cf. M a r c u s, 1971). Much less known is the relation between enzyme activity and seedlings elongation induced by GA₃ and also noted in the present work in the case of old seeds.

K a t s u m i and F u k u h a r a (1969) treating with GA₃ seedlings of dwarf maize mutants found that a-amylase activity increases parallelly with elongation. This was, however, not observed in isolated bean hypocotyls exhibiting a high a-amalyse activity (C l u m, 1967).

C o n e n et al. (1969) demonstrated that incubation of alfalfa seeds in a GA₃ solution increases proteolytic activity in the hypocotyl and cotyledons. Further investigations, however, with inhibition of endogenous gibberellins led the authors to the conclusion that gibberellic acid is not the only regulator of this process.

A series of experiments performed by Michniewicz and his collaborators (Michniewicz and Stanisławski, 1965; Michniewicz and Lampaska, 1965; Michniewicz and Kamieńska, 1969) showed that in bean seedlings growing on a medium with GA₃ added, the level of physiologically active substances rises simultaneously with hypocotyl elongation. No strict correlation could, however, be established between the growth effect and the activity of some enzyme. The results of the scarse studies in this respect indicate as it seems an indirect effect of GA₃ in connection with the enhancement of growth processes.

The most univocal results were obtained in the investigations concerning the influence of GA₃ on ribonuclease activity (cf. Key, 1969). The present investigations support his results. The rapid increase of ribonucleolytic activity is associated with r-RNA degradation and synthesis of a low-molecular weight RNA fraction detected in tissues of GA₃-treated plants (Ingle et al., 1965; Masłowski et al., 1971).

The weak reaction to GA₃ of young lupin seeds seems to be typical for *Papilionaceae* which are characterized by a high level of endogenous gibberellins (cf. Phinney and West, 1960). The experiments of Opik (1966) and Dale (1969) demonstrated that, contrary to cereal seeds, initiation and further hydrolytic activity in the first stages of incubation occur in bean cotyledons as well when the axis is absent. Treatment of these cotyledons with exogenous gibberellin for the first 3—4 days of incubation does not enhance hydrolytic enzymes activity.

To sum up, it may be affirmed that the direct effect of GA₃ on old seeds consisted in elongation of the embryonic axis, owing to which the per cent of abnormal seedlings greatly decreased. The changes in enzymatic activity accompanying this phenomenon also corresponded to changes specific for elongation. A considerable decrease of ribonuclease activity in old seeds, and its enhancement caused by GA₃ obtained simultaneously with the growth effect confirms the opinion of Grzesiuk and Kulka (1971a) that the activity of ribonucleolytic enzymes may serve as measure of the process of ageing.

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Wpływ kwasu giberelowego na aktywność enzymów hydrolitycznych
w kiełkujących nasionach łubinu różnego wieku

Streszczenie

Obserwacje przebiegu kiełkowania wykazały, że nasiona 6-letnie kiełkują w 70%, jednak więcej niż połowa tych kiełków rozwija się anormalnie. Traktowanie tych nasion GA_3 nie wpłynęło na ogólną zdolność kiełkowania, lecz obniżyło ilość kiełków wadliwych do 28%.

Aktywność α-amylazy w osiach zarodkowych i liścieniach nasion starych po 24 godzinach inkubacji była znacznie niższa w porównaniu z nasionami młodymi. W miarę dalszego kiełkowania różnica ta malała i po upływie 96 godzin była już znikoma. Inkubacja nasion z GA_3 nie zwiększyła ogólnej zawartości α-amylazy, lecz przyspieszyła wzrost jej aktywności.

Aktywność proteazy w nasionach starych i młodych była bardzo zbliżona. Traktowanie nasion GA_3 również nie spowodowało większych zmian w aktywności tego enzymu w obu partiach nasion.

Największe różnice tak w zależności od wieku, jak również od wpływu hormonu, stwierdzono w aktywności rybonukleazy, która kształtowała się odpowiednio do rozwoju kiełków. Wskazuje to, wydaje się, na pośredni wpływ GA_3, związany z efektem wzrostowym.