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Changes in nuclease activity, RNase iso-enzyme profiles and nucleic acids of germinating *Phaseolus vulgaris* L.

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Abstract. The nuclease activity in embryonic axes of *Phaseolus vulgaris* L. is by preference a RNase activity which, successively decreased and increased as germination proceeded. A direct correlation between nuclease activity and nucleic acid content was mostly non-existent in axes. The DNase activity exhibited a double strand preference during early stages of germination as opposed to a single strand preference later on. The change in RNase activity of embryonic axes during germination occurred with a change in the RNase iso-enzyme composition. After 1 h imbibition the RNase consisted of four iso-enzymes of which at least two disappeared as germination proceeded to 72 h when three iso-enzymes could be distinguished. The nuclease of the cotyledons is preferably a RNase while the inherent DNase activity showed a single strand preference at all stages of germination. A slight increase only in RNase and DNase activity of cotyledons which occurred with a minor decrease in RNA content could be observed during germination. In view of molecular genetic studies the embryonic axes of the 12 h germination period are regarded as most suitable due to a low nuclease activity.

Key words: Germination; Iso-enzymes; Nuclease; Nucleic acids; *Phaseolus vulgaris*.

Introduction

The activities of nucleolytic enzymes, mostly RNases, from a variety (except beans) of germinating seeds and seedlings have been studied and seem to be subjected to continuous changes as germination proceeds (Barker *et al.*, 1974; Beevers and Splittstoesser, 1968; Bryant and Greenway, 1976a,b; Gomes Filho and Sodek, 1988; Jacobsen, 1980; Ledoux *et al.*, 1962; Pietrzak *et al.*, 1980; Takaiwa and Tanifuji, 1978 and Wilson, 1975). Wilson (1975) mentions the absence of a direct correlation between RNase levels and RNA metabolism in plants. In general RNase activity increases and RNA content decreases in storage organs of germinating seeds as opposed to the developing axes of the germinating seedling where RNase activity and RNA content increase together. However, the development of ribonuclease activity in the cotyledons of germinating *Pisum arvense* (Barker *et al.*, 1974) and *Pisum sativum* (Bryant and Greenway, 1976a) is biphasic. Information on DNase activity in germinating seeds of any plant species is scarce. Bryant and Greenway (1976a) reported peak DNase activity during a period of net increase in DNA content in pea cotyledons.

Earlier studies with seeds reveal several iso-enzymes of RNase in cotyledons of *Vigna unguiculata* (Gomes Filho and Sodek, 1988) and *Pisum sativum* (Bryant and Greenway, 1976b and Jacobsen, 1980); embryonic axes of *Pisum sativum* (Takaiwa and Tanifuji, 1978) and seeds of the grass family (Pietrzak *et al.*, 1980; Vold and Sypherd, 1968 and Wiśniowska and Morawiecka, 1985).

Although Walbot (1971 and 1972) and Walton and Soofi (1969) studied RNA metabolism and total nucleic acid synthesis respectively during germination of *P. vulgaris*, RNase and DNase activities were not inves-

tigated. In view of our intention to experiment on the genetic transformation of *P. vulgaris*, using embryonic axes, and due to the lack of information on nuclease activity in the germinating seeds, we considered it necessary to perform this study. It may contribute to a better understanding of nucleic acid metabolism during germination of beans and definitely alleviates existing shortcomings in information on nucleic acid catabolism of germinating *P. vulgaris*, an important crop plant.

Materials and Methods

Plant Material

Phaseolus vulgaris L. cv Top Crop seeds were surface sterilised (3 min) in an 0.05% (v/v) Panacide (5,5'-2,2'-dihydroxydiphenylmethane) (BDH Chemicals) solution and washed with distilled water. The seeds were germinated for specific times on filter paper soaked with distilled water in the dark at 25°C.

Extraction of Nuclease

Ground preweighed embryonic axes and cotyledons of a known number of seeds were extracted in cold 0,3 M sodium acetate (NaAc) buffer (pH 5.5). Axes and cotyledons were respectively ground to a powder in liquid nitrogen and on ice directly in buffer with the aid of broken glass. After $(NH_4)_2SO_4$ saturation (80%) of the extract supernatants the protein precipitates were dissolved in minimum volume of 0,3 M NaAc buffer (pH 5.5), ultrafiltrated (desalting) and used for enzyme assays. For gel electrophoresis the extract was taken up in electrophoresis sample buffer after ultrafiltration.

Measurement of RNA and DNA

Ground freeze-dried axes or cotyledons were analysed for RNA and DNA (diphenylamine test) according to the method described by Cherry (1973).

Enzyme Assay

A modifified method of Keys and Zbarsky (1980) was used for the enzyme assay. Substrates for nuclease activity were highly polymerized RNA from yeast (250 μg ml^{-1}, Serva), native (ds) and thermally denatured (ss) thymus DNA (400 μg ml^{-1}, Serva). Known volumes of enzyme samples were incubated at 37°C with 150 μl substrate in 0.04 M NaAc buffer (pH 5.5) for a known period of time. After the addition of 6% (v/v) $HClO_4$ containing 0.3% (w/v) uranyl acetate to obtain a final relative concentration of 2.3% (v/v), and centrifugation, the absorbancy (A) of the acid-soluble fractions was determined at 260 nm. Readings were not corrected for dilution with perchloric acid. One unit of enzyme activity (eu) is defined as the amount of enzyme that liberates acid-soluble products with an absorbancy at 260 nm of 1 per min of reaction time under the conditions of this assay. Activities are expressed as milli-enzyme units (m.eu.) per 10 seeds or specific activity (m.eu. mg^{-1} bovine serum albumin (BSA) protein equivalent). Protein determinations were according to Bradford (1976).

SDS-polyacrylamide Gel Electrophoresis of RNases

Mini vertical gel slabs (100×75×0.75 mm) containing wheat germ rRNA (0.3 mg ml^{-1}), and buffers were prepared according to Laemmli (1970). Crystalline SDS from BDH (product No 30176) was used as recommended by Blank *et al.* (1982). Wheat germ rRNA was prepared by the method of Singh and Lane (1964). For enzyme activity the samples in sample buffer (Laemmli, 1970) without 2-mercaptoethanol, were heated prior to application for 3 min at 70°C. After a pre-run of 30 min (250V), gels were loaded and ran at constant voltage of 100 and 250V for spacer and main gels respectively. The procedures of Blank *et al.* (1982) for the removal of SDS and of Wilson (1971) for activity-staining were combined and modified. After electrophoresis gels were slightly agitated in 50 mM citrate buffer (pH 5.5) containing 25% (v/v) isopropanol which was changed 3x during a period of 30 min. After a pre-incubation period of 13 min in 50 mM citrate buffer (pH 5.5) at 37°C the gels were incubated for different periods of time, depending on the activity of the enzyme, in new citrate buffer at 37°C. Staining was performed in a solution of 0.2% (v/v) toluidine blue in 0.5% (v/v) acetic acid for 5 min under gentle shaking. Excess staining solution was washed off under running tap water followed by destaining in 0.5% acetic acid until the activity bands showed up. For total protein and standard protein (Pharmacia) separations, samples in sample buffer (Laemmli, 1970) were heated (3 min, 70°C) in the presence of 2-mercaptoethanol prior to application. Directly after electrophoresis these lanes were removed by cutting and stained for 10 min in a solution of 0.25% (w/v) Coomassie Blue R250 (Merck) in 50% (v/v) methanol and 7% (v/v) acetic acid. Gels were des-

tained in a 40% (v/v)/methanol/7% (v/v) acetic acid solution.

Results and Discussion

Nuclease Activity and Nucleic Acids in Embryonic Axes

A descending and an ascending phase in nuclease activity of embryonic axes can be distinguished over a germination period of 96 h (Fig. 1). When the enzyme activity is expressed g^{-1} dry mass (results not shown) a similar pattern to (Fig. 1B) can be observed. These tendencies were confirmed in a second experiment with a different batch of beans (results not shown). RNase activity predominates throughout this period of germination, reaching a minimum at 12 h. A similar RNase activity profile was observed during a 40 h germination period of wheat by Vold and Sypherd (1968). They produced evidence that their results indicate the presence of two RNase activities; one decreasing as germination begins and another increasing. An increasing RNase activity only, was found in the embryonic axes of peas during germination (Beevers and Splittstoesser, 1968; Takaiwa and Tanifuji, 1978). The increase in RNase activity in peas proceeds in parallel to RNA synthesis (Takaiwa and Tanifuji, 1978) and accumulation (Beevers and Guernsey, 1966). The relative high RNase activity (*in vitro*) after 1 h imbibition (Fig. 1) coincides with the low RNA synthesis rate found by Walbot (1972) and Walton and Soofi (1969) for embryonic axes (*P. vulgaris*) of comparable germination stage. They did not investigate RNase activity. These results are in agreement with the low RNA content found in axes of this particular stage of germination (Fig. 2A). This could suggest a net breakdown of RNA at the initial stage of germination. Walbot (1971) however claimed that RNA synthesized during embryogeny is maintained without degradation during the first hours of germination. The decreasing RNase activity (Fig. 1) and the slow increasing rate of RNA synthesis (Walbot, 1972) during the first 12 h of germination might have contributed to the constant RNA content observed (Fig. 2A). The rise in RNase activity after 12 h of germination (Fig. 1) is associated with RNA accumulation (Fig. 2A) which is probably due to net RNA synthesis since Walbot (1972) found a high synthesis rate. It is noteworthy that the switch from a decreasing to an increasing RNase activity occurred at a period (12 -14 h) when, according to Walton and Soofi (1969), cell division initiates. A surprisingly similar relationship between RNase activity and total RNA content was obtained by Vold and Sypherd (1968) with germinating wheat.

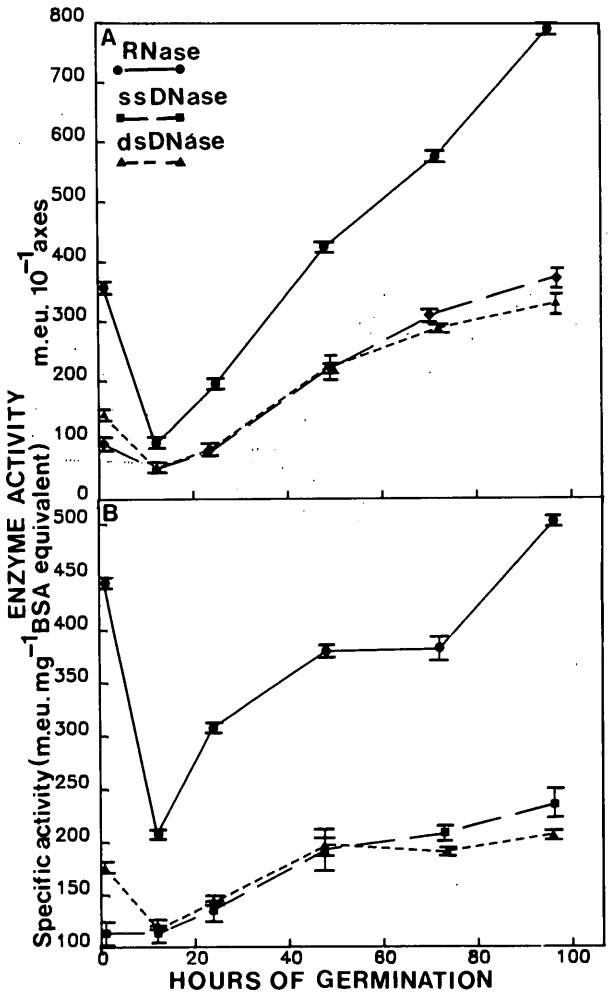

Fig. 1. Changes in nuclease activities [express in A as milli-enzyme units (m. eu.) 10^{-1} axes and B as specific activity] in embryonic axes during germination. [I = standard deviation, n = 3; coefficients of variation (CV = %) for enzyme assay are 0.7, 4.7 and 4.4 for RNA, ss and ds DNA substrates respectively, n = 16].

Strangely, the DNase activity of the enzyme did not discriminate much between double (ds) and single stranded (ss) DNA (Fig. 1) except for the higher dsDNase activity at the 1 h germination period. In contrast, the DNase activity in *Petunia* leaves (Plischke

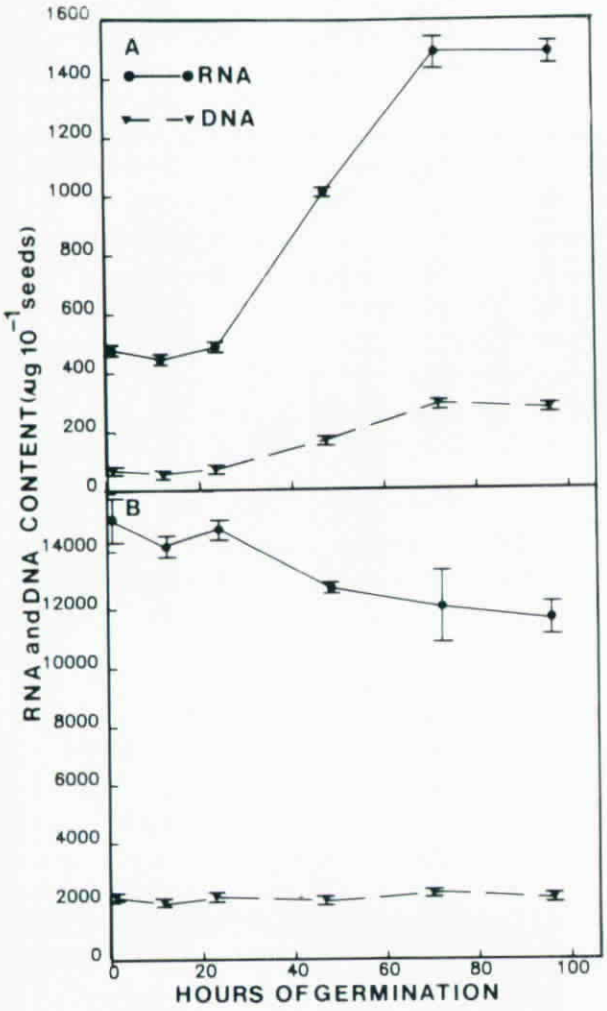

Fig. 2. Changes in RNA and DNA content in axes (A) and cotyledons (B). [I = standard deviation, n = 4].

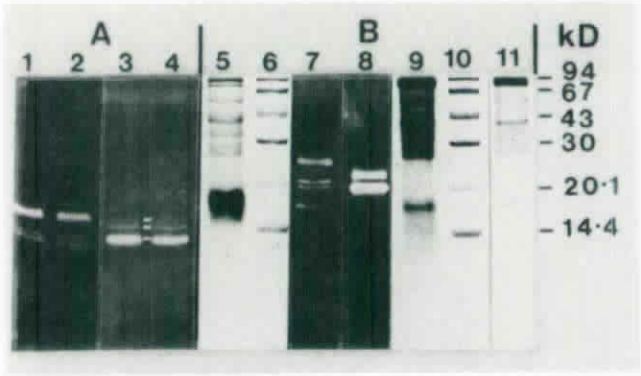

Fig. 3. RNase iso-enzyme activity bands and total protein bands of embryonic axes at 1 and 72 hours of germination in (A) 12% and (B) 15% sodium dodecyl sulphate polyacrylamide gels. Dots between lanes 3 and 4 are to indicate bands.

Lanes	Sample (hrs of germination)	μg BSA protein equivalents loaded	Reaction time at 37°C (min)
1&2	1	14.05	60
3&4	72	18.6&12.4	35
5	1 (total protein)	14.05	–
6&10	protein standards	–	–
7	1	3.76	90
8	72	4.58	90
9&11	72 (total protein)	31&18.6	

(BSA = bovine serum albumin)

and Hess, 1980) and pollen (Van der Westhuizen *et al.*, 1987) showed a relative high preference for ssDNA. An interesting phenomenon is the change from ds to ssDNA preference as germination proceeds (Fig. 1) which may be ascribed to the presence and or appearance of different nuclease molecular species with different substrate preferences. Although not for beans (*P. vulgaris*), the existence of such iso-enzymes is well documented for other plants by Bryant and Greenway (1976b), Jacobsen (1980), Matoušek and Tupý (1983), Pietrzak *et al.* (1980), Takaiwa and Tanifuji (1978) and Van der Westhuizen *et al.* (1987) amongst others. The increase in DNase activity (Fig. 1) during later stages of germination occurred with a concurrent rise in DNA content (Fig. 2A).

Iso-enzyme Patterns of Embryonic Axes

We have proved by means of SDS inhibition that the RNase activity bands depicted in Fig. 3 were real (results not shown). After 1 h imbibition the RNase activity of embryonic axes consisted of four iso-enzymes (Fig. 3, lane 7) with approximate molecular masses ranging from 27000 to 17000. The separation on the 12% gel, showing only remains of the 17000 band (front) in lane 1 was semewhat inferior to that on the 15% gel. The RNase activity after 72 h germination showed two clear bands (lane 8) of which the slow running band can be separated into two bands on the 12%

gel (lanes 3 and 4). A total of three iso-enzymes with approximate molecular masses ranging from 25000 to 20000 are therefore distinguishable. It is noteworthy that the RNase iso-enzymes of the 1 h germinating period (lane 7) representing the highest and lowest molecular masses are absent at the 72 h germination period. In addition it seems that one of the remaining two iso-enzymes of the 1 h germination period was still active after 72 h of germination. It is tempting to speculate that this activity had increased, but quantities loaded and reaction times were chosen for maximal resolution on gels only. Differential increases in iso-enzyme activity as germination proceeds have been found by Takaiwa and Tanifuji (1978) for pea embryonic axes and by Gomes Filho and Sodek (1988) for *Vigna* cotyledons. The iso-enzyme composition of the 1 h germination period therefore greatly differed from that of the 72 h germination period. The RNase iso-enzyme pattern of pea cotyledons also changed during germination (Bryant and Greenway, 1976b).

The changing iso-enzyme composition as germination proceeds could have contributed to the observed changes in RNase activity (Fig. 1). These results suggest a changing nucleic acid catabolism in embryonic axes as germination proceeds. If the individual RNase iso-enzymes posessed intrinsic DNase activities the changed composition could have played a role in the observed changes in DNase activity (Fig. 1) as well. An intrinsic DNase character is not uncommon since nucleases grouped as plant nuclease I are characterised as sugar-nonspecific (Wilson, 1975). Pietrzak *et al.* (1980) isolated three enzymes from barley seeds that can be classified as RNase I, RNase II and plant nuclease I according to Wilson (1975). Furthermore the intrinsic DNase character of pollen nucleases were found to be nonseparable by methods applied by Matoušek and Tupý (1983) and Van der Westhuizen *et al.* (1987).

Nuclease Activity in Cotyledons

The RNase character and the ss preference of the DNase activity of the nuclease through all stages of germination are clear (Fig. 4). A tendency of increasing activity as germination proceeds can be observed for both RNase and DNase which could be related to their role in the mobilization of macromolecules, i.e. nucleic acids. This tendency was confirmed in a second experiment with a different batch of beans (results not shown). Although the DNA content of cotyledons did not vary substantially there was indeed a minor decrease in RNA content as germination proceeded (Fig. 2B). The increasing RNase activity, although less striking, is consistent with the results found by Bryant and Greenway (1976a) and Beevers and Splittstoesser (1968) for pea cotyledons during the same period of germination.

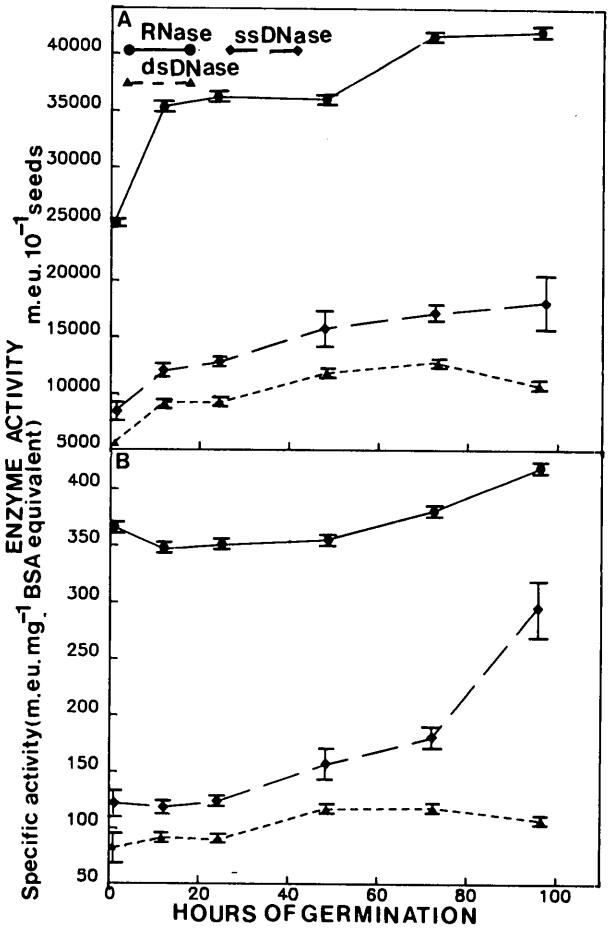

Fig. 4. Changes in nuclease activities [express in A as milli-enzyme units (m.e.u.) 10^{-1} axes and B as specific activity] in cotyledons during germination. [I = standard deviation, n = 3; coefficients of variation (CV = %) for enzyme assay are 0.9, 5.5 and 3.7 for RNA, ss and ds DNA substrates respectively, n = 16].

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菜豆 (*Phaseolus vulgaris* L.) 萌芽時核酸酶活性，核糖核酸同功酶酶譜及核酸之變化

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菜豆 (*Phaseolus vulgarid* L.) 胚軸的核酸酶在性質上偏向於核糖核酸酶 (RNase)，其活性隨著萌芽過程首先下降然後上升。核酸酶活性和核酸含量沒有直接關連。去氧核糖核酸酶 (DNase) 的活性在萌芽早期對雙股去氧核糖核酸 (DNA) 作用較強，在後期則偏向單股 DNA。萌芽時，胚軸內隨著 RNase 活性的改變，其 RNase 之同功酶組成亦發生變化，發芽後 1 小時，含有 4 個 RNase 之同功酶，在 72 小時後，其中至少有 2 種消失，而有 3 種可區別出來。在整個萌芽過程中，子葉的核酸酶偏向 RNase，而本來就有的 DNase 活性則偏向單股DNA。萌芽時，子葉內的 RNase 及DNase 活性略微增加，同時核糖核酸 (RNA) 的含量也稍微下降。萌芽 12 小時後，因爲核酸酶活性低，最適合在此時對胚軸作分子遺傳學的研究。