EFFECTS OF NATURAL AGEING ON PROTEIN COMPOSITION IN SUNFLOWER SEEDS (*Helianthus annuus* L.)

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Abstract :

Protein composition of sunflower seeds has been studied in order to evaluate seed germinability and identify a possible marker for germination capacity. Protein composition was analyzed at different levels ; 2-D gels electrophoresis was used to appreciate total soluble proteins, *in vivo* and *in vitro* synthesized proteins in seed lots showing different germinabilities.

Higher differences were observed in *in vivo* and *in vitro* protein patterns as well as in the rate of protein synthesis. Methionine uptake and its incorporation were strongly reduced in low germinability seeds. A net decrease have been also shown in RNA content of the dry seed and in the ability of RNA to be translated *in vitro*.

Germination is affected at several levels including membrane, enzymatic and nucleic acid deteriorations.

Introduction :

Diminution of germination capacity of seed lots has already been correlated with several parameters such as insect or fungus attacks (HALLOIN, 1986), morphological parameters like seed weight or seed size, or biochemical events like degradation of DNA and RNA (WILSON and MAC DONALD, 1986), alteration of protein synthesis (DELL'AQUILA, 1986, 1989) or membrane properties deterioration (SIMON and RAJA-HARUN, 1972). Nevertheless, discrimination of more precocious events that could lead to loss of the seed germinability and then affect seed lot germinability is difficult. Differences in seed lot quality can appear not only at the final percentage of germination level, mean germination time level, but include vigor seedling, normal or abnormal seedlings and final yield too. Yet, it is not possible to impute a precise signal to these different manifestations of seed quality loss.

An important event in germination process seems to be the early phase of imbibition. Under optimal conditions, seed imbibition and metabolic reactivation occur leading to new synthesis. Recent studies have shown that decrease in seed viability could be linked to reduction in protein synthetic capacity (GIDROL *et al.*, 1988 ; REUZEAU *et al.*, 1992), decrease in RNA content in the dry seed (NOUBHANI, 1990 ; REUZEAU, 1992), and in the first hours of imbibition (BRAY and SMITH, 1985 ; BLOWERS *et al.*, 1985 ; THOMPSON *et al.*, 1987). In this paper, we examine protein composition and protein synthesis capacity in relation to germinability of non dormant sunflower seed lots. Protein content, RNA amount, protein and RNA synthesis capacity were determined ; what of these elements could be essential for germination to be completed successfully is researched too.

MATERIAL AND METHODS.

Plant material, imbibition and germination test.

Sunflower seeds (Helianthus annuus L.) originate from the same simple homogene hybrid. Two seed lots, harvested for 12 months, with germination rates of 92 and 74% are used for all experiments. Seeds were placed on 4 layers of water-imbibed filter paper at 19°C at a light intensity of

100 µE/m2/s, and 14/10 hours (day/light) photoperiod. Germination is defined as 1 mm radicle emergence and followed for 7 days.

In vivo protein synthesis and extraction of proteins.

After imbibition (0, 6 or 12 hours), seeds are decorticated and cotyledons and embryonic axes are separated. These organs are individually placed in microfuge tubes in labelling solution containing 35 S-Methionine (7, 3.36 and 0.67 MBq/100µl distilled water for 0, 6 and 12 hours imbibition respectively, Amersham, 37 TBq/mmol) for 4 hours at 19°C. Samples are then washed, dried and homogeneized in a solution containing 9.2 M urea, 0.5% chapso, 3% nonidet P40, 1% triton X100 and 0.5% dithiothreitol. Extracts are then centrifuged at 15000 x g for 15 min. Protein content is determined using the Bradford assay (BRADFORD *et al.*, 1976). Radioactivity uptake and incorporation into the trichloroacetic precipitable fraction are measured.

In vivo RNA synthesis and extraction of RNA.

This method has been described by STOILOV *et al.*(1989) and modified for sunflower seeds. 100 seeds are separated and organs are then incubated in presence of 3H-Uridine (1850 KBq/500µl distilled water ; Amersham, 1.3-1.8 TBq/mmol) for 2 hours at 19°C. Organs are then rinsed in sterile water and grinded in liquid N2. Fine powder obtained is then resuspended (500µl for 0.2g of fresh material) in tris-acetate 100mM pH9, containing NaCl 100mM, EDTA 1.5mM and SDS 5% (HAFFNER *et al.*, 1978). The RNA concentration was determined spectrophotometrically at 260 nm. Newly synthesized RNA are determined using liquid scintillation spectrometry.

In vitro translation of RNA

Total RNA (15µg) is incubated in presence of 35µl rabbit reticulocyte lysate (Promega), 1.12 MBq 35 S-Methionine, 2µl RNasin (Promega) and 2µl amino-acids during 1h30 at 30°C. The reaction is stopped by addition of a mixture containing 2.5µl EDTA 250mM, pH8 and 2.5µl RNase A (0.1 µg/µl) +T1 (60mU/µl) and incubation during 30 min at 30°C. Proteins are then precipitated at 0°C for 1h by addition of 16 volumes 20% TCA-acetone. Pellet is washed by methanol and solubilized in lysis buffer. Proteins were then analysed by two-dimensional PAGE.

Two-dimensional electrophoresis of proteins (NEPHGE).

First dimension is performed according to MEYER *et al.* (1988) in cylindrical gels. After migration and equilibration, gels are then placed on the second dimension 13.5% acrylamide gel according to LAEMMLI (1970). Gels are stained with coomassie brillant blue R250 ; labelled proteins are detected by autoradiography after fluorography (Amplify Amersham).

RESULTS.

Total proteins of dry seeds.

Changes in protein profiles between dry seed lots of different germinative capacity were analysed by two-dimensional electrophoresis. Significative differences between protein patterns are essentially located in the 3 areas a (18-22 kDa ; pI=5), b (20-25 kDa ; pI=6.5) and c (25-30 kDa ; pI=5) (data not shown) ; they are observed mainly in the low germinative capacity seeds. However, observation of storage proteins between seed lots indicate no differences neither qualitative nor quantitative associated

with germinative quality. So, analysis of total proteins by 2D-electrophoresis was insufficient to characterize significantly seed lots in terms of germinability.

Newly synthesized proteins in the early phases of germination.

During early imbibition, different protein patterns can be observed between seed lots of different germinability. At 0h of imbibition, during the 4 h of label, several proteins are specific of seeds which are able to germinate. Figure 1A shows polypeptides synthesized only in the cotyledons providing from the high germinability seed lot. Some of them could be imbibitional peptides, induced only at this time, and implicated in the germination process After a 6 hours imbibition period, some of the precedent proteins are still synthesized (Figure 1B). Not all of the proteins of the precedent pattern maintain their synthesis. Several new polypeptides are synthesized in cotyledons and in embryonic axes. Some of these proteins seem to be specific of high germinability seed lot. After 24h of imbibition, proteins differ between seeds of different germinability (REUZEAU *et al.*, 1992) but such variations could be more significantly attributed to developmental processes.

In the same time for seeds showing low germinative capacity, rate of *in vivo* protein synthesis is severely decreased. Not only uptake of ^{35} S-Methionine but also its incorporation into proteins are reduced. Seeds have lost their ability to translate efficiently mRNA *in vivo*. Figure 2 indicates that after 6 hours of imbibition, 52.5% of the total radioactivity is absorbed for the high germinability seed lot, where as only 33.4% is absorbed for low germinability seed lot. For incorporation, values are respectively of 9.4% and 1.3%. These variations can be observed at both the embryonic axis and whole seed level. A shift appears between equivalent stages of protein mobilization in such seed lots ; it is esentially due to uptake processes and could concern germination initiation phase. Rate of protein synthesis is reduced by 80% at 6h of imbibition. This result indicates a proper deficiency of incorporation not exclusively due to absorption. This can so implicates membrane and enzyme deterioration processes. Rate of protein synthesis is reduced after 24h of imbibition too ; it can be correlated with germination percentage (figure 3). Results are different from those obtained during accelerated ageing suggesting *in vivo* protein synthesis is not affected in sunflower seeds (GIDROL *et al.*, 1990)

Changes in RNA and *in vitro* protein synthesis.

New data can be brought by *in vitro* protein synthesis from dry seed RNA or newly synthesized RNA at the onset of imbibition. Significant changes in total RNA have been observed. A high correlation appear between amount of total RNA and seed germinability (Figure 4). Moreover, decrease in not compensated during imbibition (data not shown). Rate of new RNA synthesis is reduced as early as 2h of imbibition for a low germinability seed lot ; this diminution still persists after 24h of imbibition. It can be so correlated with seed moisture content at the harvest (figure 5). Once again, uptake mechanisms (3H-uridine) as well as incorporation processes are concerned.

Analysis of translation products by 2D-electrophoresis reveals five specific polypeptides of high germinability seeds (Figure 6). They have molecular weights of 10 kDa (pI=7), 14 kDa (pI=6.5), 31 kDa (pI=7.3), 94 kDa (pI=5) and 100 kDa (pI=6.7). Nevertheless, none of them has been yet characterized. Polpeptides of 94 kDa and 100 kDa are induced *in vivo* after 4h of imbibition, indicating that they could be imbibitional peptides. In conclusion, some of the early synthesized polypeptides are not present in the seeds exhibiting low germinative capacity.

DISCUSSION.

Dry seeds contain stored mRNA that play a predominant role in governing proteins synthesis during the initial stages of germination (DELSENY *et al*., 1986). If mRNA or proteins contained in the dry seed represent a prerequisite for protein synthesis at the onset of imbibition, dry seed quality and seed germinability could be so correlated.

Seed lots of different germinability can be distinguished by their protein composition in the dry seed as well as during the first hours of imbibition. The ability to synthesize new proteins at this time , since four hours of imbibition, is highly reduced, especially in seeds which are not able to germinate. They lack of several proteins or their synthesis is delayed and newly polypeptides can not be observed on the fluorograph. Some proteins could be so essential for successfull germination. At this time, they can not be identified and their precise function remains unknown. Polypeptides detected only during early germination, proceeding from the dry seed mRNAs, can characterize late embryogenesis and could be regulated by ABA or osmotic stress. Other new synthesized proteins are observed after 6h of imbibition ; they result from newly synthesized mRNA. Other different proteins, present after 24h of imbibition would be proteins implicated in the growing phase.

CONCLUSION.

Natural ageing could affect protein synthesis, especially during early germination, and so reverberate on germinative capacity, mean time of germination and seedling growth. In an other way, characteristics of protein synthesis are highly reduced for seed which are unable to germinate. Reduction of protein synthesis is affected at the membrane level (uptake processes) and the proteic level (incorporation processes). Decreased protein synthesis capacity may be linked to lower dry seed RNA content and integrity and also reduced RNA synthesis capacity. We can so consider early initiation mechanisms of germination and late developmental mechanisms and so restrict to the first germinative capacity problems.

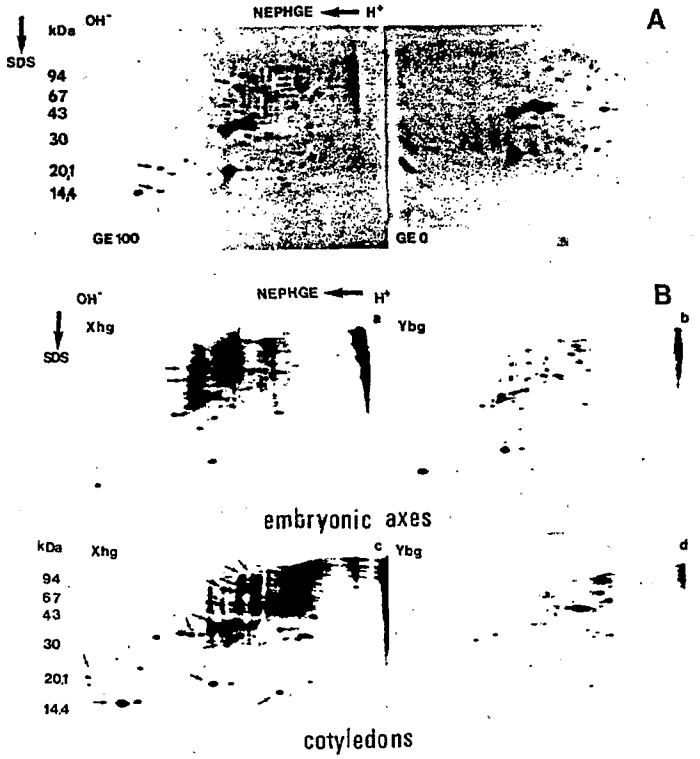

Figure 1 : Autoradiograms of proteins synthesized in vivo at the onset of imbibition (A : 0h ; B : 6h). Proteins were labelled with 35S-methionine during 4h and separated by NEPHGE-SDS-PAGE electrophoresis. Proteins were analysed in cotyledons (A) and in cotyledons (c,d) and embryonic axes (a,b) (B), in high (GE 100 ; Xhg) and low (GE 0 ; Ybg) germinability seeds. Molecular weights of the marker proteins are indicated on the left. Arrows indicate specific proteins of high germinability seeds.

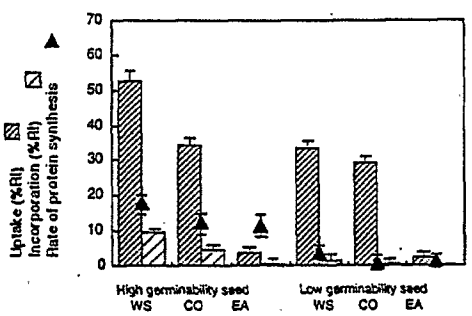

Figure 2 : Uptake of 35 S-methionine ( ), incorporation ( ) and rate of protein synthesis ( ) after 6h of imbibition in two different germinability seed lots. Values of uptake and incorporation are given as a percentage og initial radioactivity (RI). Results are the means of 5 experiments.
WS : Whole seed ; CO : Cotyledons ; EA : Embryonic axes.

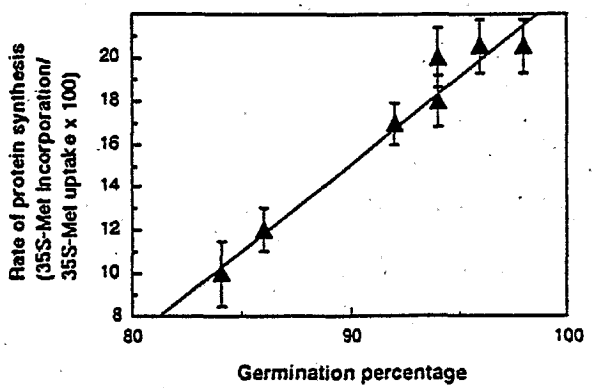

Figure 3 : Relation between percentage of germination and rate of protein synthesis after 24h of imbibition. Results are the means of 4 experiments.

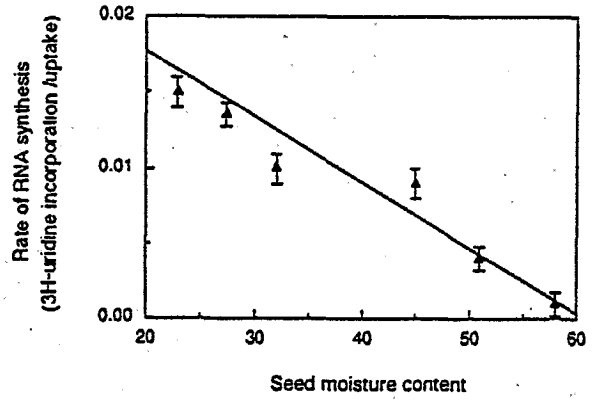

Figure 5 : Relation between rate of RNA synthesis after 24h of imbibition and seed moisture content at the harvest. Values are the means of 4 experiments.

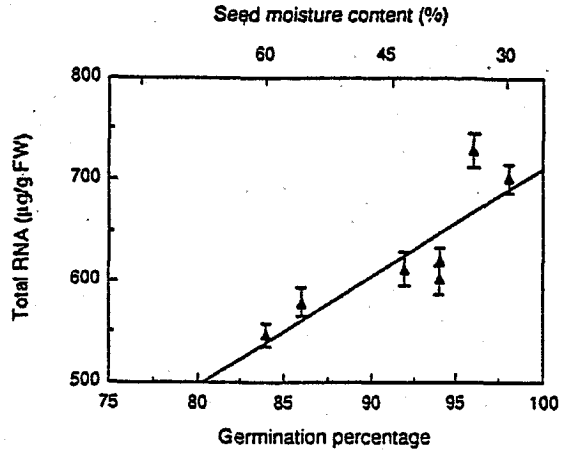

Figure 4 : Relation between amount of total RNA in the dry seed and seed germinability. Values are the means of three experiments.

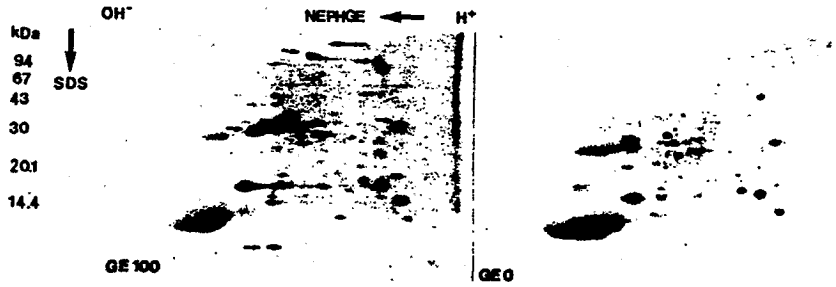

Figure 6 : In vitro translation products of RNA extracted from dry seed cotyledons of high (GE 100) and low (GE 0) germinative capacity. Proteins are separated by NEPHGE-SDS-PAGE electrophoresis. Marker proteins are indicated on the left. Arrows indicate specific proteins of high germinability seeds.

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