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Effect of desiccation on isolated embryos of maize. Onset of desiccation tolerance during development

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SUMMARY

The research described in this paper was designed to define the stage of development during which the excised embryo of maize acquires desiccation tolerance. The applied desiccation stress corresponded to a period of 2 days at 35 °C (during which the isolated embryos reached a water content around 3.8 %). The experiments led to the following conclusions : at stages where precocious germination is fully evident, the embryo can still be desiccation-intolerant ; — desiccation tolerance is acquired by the embryo at a very precise developmental stage, between 20 and 25 days after pollination (DAP) during which the dry weight increases by a factor of 5 to 10. In terms of DAP the onset of desiccation tolerance was found reproducible for 2 years and for the 4 genotypes tested. Normal development of the coleoptile and embryonic root originating from a dried embryo depends on the stage of excision : the younger the embryo is desiccated, the more abnormal are the coleoptile and the embryonic root originating from it. Acquisition of desiccation tolerance and of insensitivity of the embryo to desiccation damage is probably the result of two independent physiological processes : the first property is acquired rapidly and at earlier stages than the second. A set of proteins has been identified that is specifically expressed only at stages of development when the embryo is desiccation-tolerant. This may open possibilities for approaching the biochemistry of desiccation in the maize embryo.

Additional key words : *Seed development, resistance to desiccation, germination.*

RÉSUMÉ

Effet de la dessiccation sur l'embryon isolé de maïs. Acquisition de la tolérance à la dessiccation pendant le développement.

Ce travail a pour objectif de préciser la phase de développement pendant laquelle l'embryon de maïs devient tolérant à la dessiccation. Le stress de dessiccation appliqué correspondait à une période de 2 jours à 35 °C pendant laquelle les embryons isolés atteignaient une teneur en eau de 3,8 p. 100. L'expérimentation a conduit aux conclusions suivantes : dans le cas de germination précoce, l'embryon peut être intolérant à la dessiccation, cette tolérance est acquise par l'embryon à un moment précis du développement, entre le 20e et le 25e jour après la pollinisation (DAP). Pendant cette période de 5 jours, le poids sec est multiplié par un facteur de 5 à 10. En terme de « DAP », l'apparition de la tolérance à la dessiccation s'est révélée reproductible pendant 2 ans et pour les 4 génotypes testés. L'aptitude du coléoptile et de la radicule à effectuer un développement normal dépend du stade de développement : la probabilité pour ces organes de se développer normalement s'accroît au cours du temps après la fécondation. L'acquisition de la tolérance à la dessiccation et de l'insensibilité de l'embryon aux dégâts de la dessiccation est probablement le résultat de deux processus physiologiques indépendants : la première propriété est acquise rapidement et à des stades plus précoces que la seconde. On a identifié un groupe de protéines qui est exprimé spécifiquement seulement au stade de développement auquel l'embryon est tolérant à la dessiccation. Ce résultat peut ouvrir des possibilités pour une approche biochimique de la dessiccation de l'embryon de maïs.

Mots clés additionnels : *Développement de la graine, résistance à la dessication, germination.*

I. INTRODUCTION

During development the acquisition of tolerance to desiccation confers the ability of the seed to germinate upon drying and rehydration. In this respect drying is considered to be a beneficial event (MITCHELL *et al.*, 1978) which regulates the transition from seed development to seed germination (discussed in the introduction of the paper by KERMODE & BEWLEY, 1985 and in DASGUPTA & BEWLEY, 1982). Tolerance to water stress is not, however, a trait expressed at all stages of a developing seed ; specific stages are known during which the seed becomes desiccation-tolerant (see, for example, KERMODE & BEWLEY, 1985, for *Ricinus communis* and LONG *et al.*, 1981 and DASGUPTA *et al.*, 1982 for *Phaseolus vulgaris*).

In this paper we describe experiments performed with isolated embryos of maize to determine the developmental stage at which the onset of desiccation tolerance takes place. The protein pattern of the embryo, before and soon after the onset of desiccation tolerance, is also reported as revealed by 2D-electrophoresis. Data are, moreover, presented on the effect of the drying treatment on the germination of dissected embryos and development of the seedlings. Excision of developing embryos from the endosperm under aseptic conditions and their subsequent germination on an artificial medium allows precise determination of the onset of desiccation tolerance in the absence of the endosperm.

II. MATERIALS AND METHODS

A. Maize materials

In the first experiments (1984) described in this paper inbred maize lines B73 and Mo17 were used, while in the second (1985) the experimental material consisted of selfed seeds from the hybrid B73 × Mo17. Maize plants were grown according to agrotechnical conditions similar to those specified in the paper by GENTINETTA *et al.* (1982).

In the 1984 experiments, plants of the two inbreds were grown in Bergamo, Northern Italy, selfed and each reciprocally crossed, to give hybrid seeds with the pedigrees B73 (female) × Mo17 (male) and Mo17 (female) × B73 (male). Selfed and crossed cobs were harvested for all genotypes at 20, 25, 30, 35, 45 days after pollination (DAP) and at physiological maturity (black layer maturity, BLM). For line B73, selfed cobs were also harvested at 14, 16, 18 and 22 DAP.

In the 1985 experiment, F2 plants from the cross B73 × Mo17 were grown in Florence, Central Italy, and selfed cobs harvested at 20, 25, 30, 35, 40, 45 DAP and at BLM.

Mean daily temperature and mm of rain were recorded for both years during the period of maize growth and are reported (fig. 1) separately for the two locations. In the inserts of this figure are indicated the growing degree-day units (GDD) available to the embryos from pollination to excision. The GDD units were calculated according to BROWN (1969).

B. Embryo excision and drying

For each genotype and stage of development, embryos were aseptically isolated from 2 cobs (1984) or 4 cobs (1985). Embryos from one cob were always kept separate and considered as an independent replicate. For the drying treatments, embryos were laid between two disks of filter paper within a sterile petri dish and incubated in an oven at 35 °C for 48 h with the lid open. At the end of the desiccation period, the dishes were fixed with parafilm and conserved at 5 °C on silica gel in a desiccator until embryo germination. The embryos isolated in 1984 were stored for 9 to 12 months and for 19 months respectively before germination experiments and plantlet developmental experiments. The embryos isolated in 1985 were stored for 2 months before use. Water content of dried embryos was determined after desiccation at 90 °C for 48 h and averaged around 3.8 % (in the experiment of 1984 3.7, 4.33, 2.03, 5.29 and 3.69 % respectively for 20, 22, 25, 30 and 45 DAP).

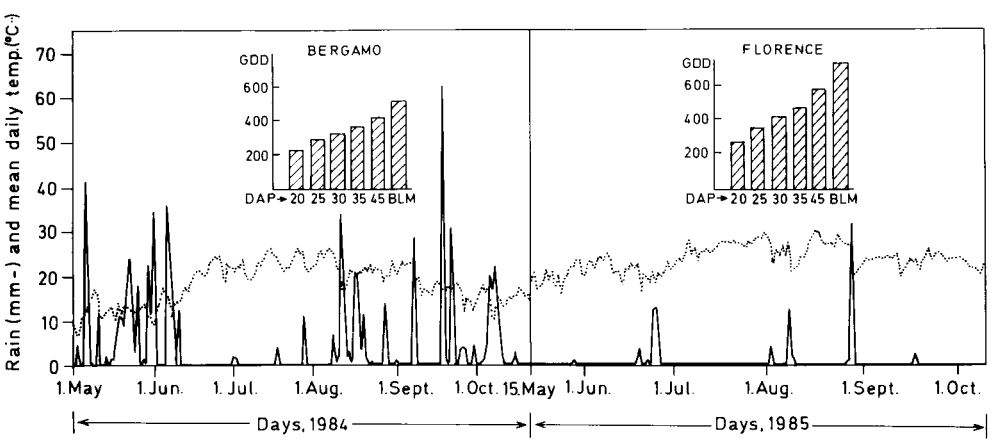

Figure 1

Mean daily temperature (°C), — mm rain from emergence to BLM time and, (inserts) growing degree days units (GDD) cumulated from pollination to the time of excision of embryos in 1984 (location Bergamo) and 1985 (location Florence).

Température moyenne journalière (°C), — mm de pluie et (insert) degrés jours cumulés (GDD) de la pollinisation à l'excision des embryons pendant les années 1984 (Bergamo) et 1985 (Florence).

C. *In vitro* embryo germination

Fresh or dried embryos were germinated on an artificial solid medium containing in 1 000 ml of H_2O : 0.6 g of $(NH_4)_2NO_3$, 0.4 g of $MgSO_4$. 7 H_2O, 0.4 g of KH_2PO_4, 0.16 g of K_2HPO_4, 0.006 g of Fe citrate. 5 H_2O, 0.4 g of $CaHPO_4$, 20 g of sucrose and 8 g of agar. Embryos were germinated at 25 °C in the dark in lots of 5 per 9 cm petri dish containing 20 ml of medium. In the petri dish the embryonic axes of the embryos were positioned upward to make the evaluation of incipient germination possible. In each dish embryo germination was monitored every 24 h for 10 days. An embryo was considered to have germinated when the embryonic root or the coleoptile were visibly grown (longer than 0.2 cm). For each stage and genotype the percentage of germinated embryos (PG) was calculated after 10 days. Four days after germination the percent of abnormal seedlings (PA) (abnormals : coleoptile broken or not developed or twisted ; absence or short or twisted embryonic root, poor development of both coleoptile and embryonic root), the percent of seedlings lacking only one or both seminal roots (PWSR), the percent of normal seedlings (100-PA-PWSR) were also calculated.

D. Plantlet evaluation

To determine the effect of drying or of the stage of excision of the embryo on the morphology of the derived plantlets, a specific experiment was performed. In this case individual embryos were placed in test tubes (2 × 25 cm) containing 10 ml of culture medium and incubated at 25 °C in the dark. After 24 h from germination, the tubes were moved to an illuminated cabinet (10 000 lux) with a 12 h photoperiod. Abnormal plantlets were those incapable of developing beyond the coleoptile stage or unable to produce more than 2 leaves. (In this paper we have adopted the term seedling to indicate the developing shoot and root at the coleoptile stage, while plantlet refers to the three-leafed stage).

E. 2-D-electrophoresis

1. *Protein extraction*

For each time point proteins were extracted from 10 maize embryos as described by DAMERVAL *et al.*, 1986. 25 µl of a protein extract were used for 2-D-electrophoresis. For the first dimension the proteins were separated on 18 cm long isoelectro-focusing (IEF) rod gels. The gel mixture was 4.2 % acrylamide, 0.2 % N,N′-methylenbisacrylamide, 9 M urea, 3 % NP-40, 6 % carrin ampholytes (LKB) consisting of a mixture of 1/5th Ampholine pH 3,5-10, 2/5th Ampholine pH 4-6, 1/5th Ampholine pH 5-8, 1/5th Ampholine pH 7-9. IEF was performed with 14 000 Vh. In the second dimension, the focused proteins were separated on a 7.5-15 % polyacrylamide gel overlaid with a stacking gel of 4 % acrylamide (LAEMMLI, 1970). After the tracking dye (bromophenol blue) had run to the bottom the gels were fixed in propanol-2 (25 % v/v)-acetic acid (10 % v/v) and then silver-stained according to MORRISSEY, 1981.

III. RESULTS

A. Experiment of 1984

Samples of embryos of the inbred line B73 excised at 14, 16, 18, 20, 22, 25, 30, 35 DAP and at BLM were germinated both fresh and after drying. Only the percentage of germination was recorded ; the results are presented in table 1. At the earlier stages sampled (14 DAP), fresh embryos showed a germination capacity of 87 % which at 16 DAP was already equal to 100 %. The result indicated that excised but not dried embryos of maize can precociously germinate at very early stages of development. However, rapid desiccation imposed on the embryos inhibited their germination if they were excised before 25 DAP. At 25 DAP the germination capacity after drying was almost equal to 100 %.

TABLE 1

Germination capacity of fresh and dried embryos of the inbred line B73 excised at various days after pollination (BLM = black layer maturity).

Capacité germinative des embryons frais et desséchés de la lignée B73 excisés à différents jours après la pollinisation (BLM = point noir).

	Fresh		Dried	
DAP	No.	% Germinated	No.	% Germinated
14	8	87	50	0
16	100	99	50	0
18	29	100	50	0
20	100	100	50	0
22	100	100	50	0
25	100	100	50	96
30	100	100	50	100
35	60	100	50	100
BLM	100	100	50	100

A group of 4 genotypes was further tested to confirm the results obtained with the inbred B73 and to study accurately the effect of drying on the expression of seedling and plantlet traits. Dried embryos from the genotypes B73, Mo17, B73 × Mo17 and Mo17 × B73 were prepared in the summer of 1984 at 20, 25, 30, 35, 45 DAP and at BLM and stored at 5 °C in a desiccator. The germination experiment started in June 1985 and was completed in September of the same year. Each genotype at each stage of development was represented by 40 to 140 embryos distributed in 2 replications. The results are presented in figure 2. The data of figure 2A dealing with germination percentage (PG) clearly confirmed the results of table 1 : the 4 genotypes (which did not significantly differ when their germination values were analyzed according to a factorial design involving stages of development besides genotypes) were unable to germinate if harvested and dried at 20 DAP, while at 25 DAP they showed already a mean germination percentage aroung 60 %. The effects of excision and drying on traits of the germinating seedlings are reported in figure 2B, C, and D. It is apparent that the

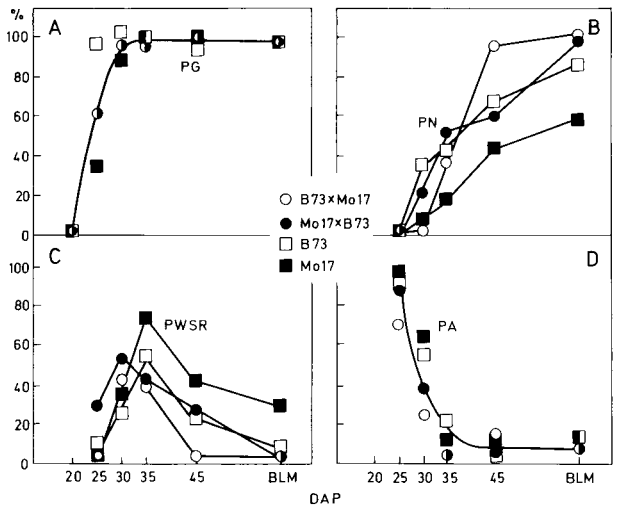

Figure 2

Percent of embryos germinated, PG (A), percent of normal seedlings, PN (B), percent of seedlings without seminal roots PWSR (C) and percent of abnormal seedlings PA, (D), in relation to number of days from pollination to excision of the embryo from the endosperm (DAP). Experiment of 1984. The embryos were excised and dried before germination on artificial medium. BLM = black layer maturity. A and D : the differences among genotypes were not significant according to a factorial design which considers both genotypes and stages of development : only one line has then been used to interpolate the data. B and C : significant differences were found among genotypes at 1 % and 5 % levels respectively for B and C ; each genotype is represented with its own trend.

A : pourcentage d'embryons germés (PG) ; B : pourcentage de plantules normales (PN) ; C : pourcentage de plantules sans racines séminales (PWSR) ; D : pourcentage de plantules anormales (PA) en fonction du nombre de jours séparant la pollinisation de l'excision de l'embryon (DAP). Expérience 1984. Les embryons furent excisés et desséchés avant la germination sur un milieu artificiel. BLM = point noir. A et D : les différences entre les génotypes ne sont pas significatives selon un schéma factoriel qui considère soit les génotypes, soit les stades de développement : une seule lignée a été employée pour l'interpolation des données. B et C : des différences significatives ont été relevées entre génotypes au niveau 1 p. 100 et 5 p. 100 respectivement.

4 genotypes reacted in a different way when the percentage of normal seedlings (PN) germinating after drying was considered as a function of the stage of the excised embryo. As a rule the nearer the embryo is to its physiological maturity, the more it is capable of producing a normal seedling, with the hybrid B73 × Mo17 normalizing before the other genotypes. When the percent of seedlings without seminal roots (PWSR) was considered, again a significant difference among genotypes was evident. This trait, moreover, showed the higher value at an intermediate stage of development for all genotypes. Abnormal seedlings (PA) were extremely abundant at 25 DAP indicating that excision and drying at this stage mainly result in abnormal seedlings, although the embryo can easily resist desiccation. For this trait no differential effects were noted for the tested genotypes.

The results reported in figure 3 support the data of figure 2D. When the normality of the plantlets originated from dried embryos was considered, it was evident that at 25 DAP only 20 % of the total germinating embryos were capable of producing a normal plantlet. At 30 DAP, however, normal plantlets were in the range of 50 % of those which germinated.

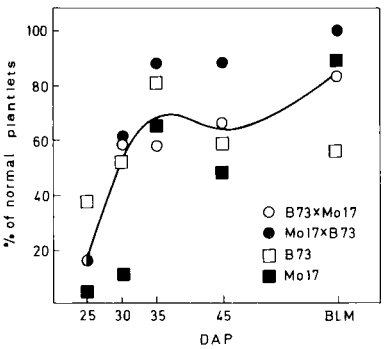

Figure 3

Percent of normal plantlets from germinated seeds in relation to number of days from pollination to embryo excision (DAP). Experiment of 1984. The embryos were excised and dried before germination. BLM = black layer maturity. The differences among genotypes calculated as in figure 2 were not significant.

Pourcentage de plantules formées en fonction du nombre de jours entre la pollinisation et l'excision de l'embryon (DAP). Les embryons ont été excisés et desséchés avant la germination. BLM = point noir. Les différences entre génotypes, calculées selon le protocole décrit dans la figure 2, ne sont pas significatives.

In conclusion the 1984 experiment indicated that the developmental step between 20 and 25 DAP was absolutely critical in determining the germinating response of dried embryos. At these stages the weight of the dried embryo increased from 0.5 to 6 mg (table 2).

TABLE 2

Mean weight of the dry embryo (mg) at 20 and 25 DAP for the 4 genotypes considered. Experiment of 1984.

Poids moyen de l'embryon desséché (mg) à 20 et 25 DAP pour les 4 génotypes pris en considération.

	Genotype			
DAP	Mo17	Mo17 × B73	B73 × Mo17	B73
	mg (± s.e.)			
20	0.39 ± 0.08	0.47 ± 0.09	1.15 ± 0.14	1.76 ± 0.20
25	2.78 ± 0.33	5.51 ± 0.21	4.86 ± 0.31	6.49 ± 0.12

B. Experiment of 1985

In this year, only the F2 generation from hybrid B73 × Mo17 was considered. The choice was consistent with the possibility of growing a representative population of the 4 genotypes tested in 1984 and with the need to restrict the experiment to only one population. The experiment was designed to test independently the effects of drying and stage of excision on embryo germination. The results are reported in table 3.

Considering first the embryos germinated when still fresh, it was evident that already at 30 DAP both their germination capacity and the traits expressed by the seedlings after germination were almost identical to those recorded at BLM. The dried embryos behaved differently. They could apparently germinate as well as

TABLE 3

Percent of embryos germinated (PG), percent of normal seedlings (PN), percent of seedlings without seminal roots (PWSR) and percent of abnormal seedlings (PA) as a function of the number of days from pollination to excision of the embryos from the endosperm (DAP). Experiment of 1985. Excised embryos were germinated as such (fresh = F) or after drying (D). Comparisons of fresh and dried embryos were made by χ^2 test according to a contingency table 2 × 2. (BLM = black layer maturity.)

Pourcentage d'embryons germés (PG), pourcentage de plantules normales (PN), pourcentage de plantules sans racines séminales (PWSR) et pourcentage de plantules anormales (PA) en fonction du nombre de jours séparant la pollinisation de l'excision de l'embryon (DAP). Expérience de 1985 : les embryons excisés furent germés tels quels (frais = F) ou après dessiccation (D). La comparaison entre les embryons frais et desséchés a été faite avec le test de χ^2 suivant la table de contingence 2 × 2. (BLM = point noir.)

DAP (1)	PG			PN			PWSR			PA		
	D	F	χ^2	D	F	χ^2	D	F	χ^2	D	F	χ^2
25	70	100	16.9****	14	50	20.5****	27	44	3.4 ns	59	6	35.8****
30	100	100	ns (2)	21	100	95.9****	62	0	59.2****	16	0	8.7**
35	100	100	ns (2)	63	93	20.8****	26	6	10.9***	12	1	5.6*
40	98	96	0.1 ns	69	95	20.5****	19	2	8.7**	12	3	2.7 ns
45	100	100	ns (2)	84	96	6.0*	1	0	0.1 ns	16	4	6.0*
BLM	99	100	0.05 ns	99	93	2.0 ns	0	5	1.9 ns	1	2	0.03 ns

*, **, ***, **** = significant respectively for P ≤ 0.05, 0.01, 0.001, 0.000 5.

(1) As in the experiment of 1984, at 20 DAP all dried embryos did not germinate. The comparison between D and F was then not considered.
(2) The value of χ^2 is not computable.

those which had not been desiccated (with the exception of the 25 DAP stage where the dried embryos had a PG value of 70 significantly different from 100, the PG value of the fresh embryos). In contrast, a strong negative effect of drying was observed considering PN, PWSR and PA. For these parameters a clear trend could be observed, i.e. the younger the embryos are excised the more the drying negatively affects the seedling characteristics. The state of the seedling originating from a dried embryo seems dependent on the stage of excision : abnormalities in seedling development can, in this respect, be evaluated only if the embryo has previously been desiccated. Moreover, the normal behaviour of the seedling after embryo desiccation seems physiologically independent from the acquisition of desiccation tolerance.

C. 2-D-electrophoresis

Polypeptides present in fresh and dried embryos at different stages of embryogenesis (15, 20, 25, 30 DAP) have been characterized by their migration in two-dimensional gel electrophoresis. Major changes in the protein pattern were observed between 20 and 25 DAP. When the protein patterns of different stages were compared, several new protein spots could be identified, present in 25 and 30 DAP but not in 15 and 20 DAP (fig. 4). Most of these proteins increased in quantity during maturation. No major qualitative differences were observed between the protein patterns of fresh and dried embryos.

IV. DISCUSSION

Embryos excised from the seed during development and cultured in nutrient media may increase in size or germinate precociously to produce seedlings. Precocious germination of detached embryos has been reported for several plants, cereals included (MERRY, 1942 ; RIJVEN, 1952 ; KLEIN & POLLOCK, 1968 ; ANDREWS & SIMPSON, 1969 ; NORSTOG, 1972 ; DURE, 1975 ; MONNIER, 1976 ; CROUCH & SUSSEX, 1981 ; LONG *et al.*, 1981 ; ARMSTRONG *et al.*, 1982 ; DURE *et al.*, 1983). Maize is a species capable of precocious germination : its embryos separated from the endosperm and cultured on an artificial medium germinate rapidly even when excised very early during development (14 DAP in this paper ; they germinate even at 8 DAP according to observations made by one of us, F.S.). For germination they apparently do not need the shock induced by drying which is frequently found necessary to induce the metabolic switch from development to germination (see KERMODE & BEWLEY, 1985 and KERMODE *et al.*, 1985 for an accurate description of the behaviour of the seed of *Ricinus communis*, a species requiring desiccation to initiate germination).

The experiments described in this paper establish that while the capacity for precocious germination is a characteristic feature acquired early by the developing embryo of maize its positive response to a desiccation stress is not acquired simultaneously but later in the development. Drying of premature maize embryos, in fact, reveals that desiccation tolerance is acquired between 20 and 25 DAP. In this short period of 5 days the percent of germination increases from 0 to 60-100 for the 4 genotypes considered. It can be concluded that maize behaves like other species, acquiring desiccation tolerance several weeks before seed maturation (ADAMS & RINNE, 1981 ; DE KLERK, 1984 ; KERMODE & BEWLEY, 1985 ; LALONDE & BEWLEY, 1985). During this period the embryo dry weight of maize increases by a factor of 5 to 10 depending on the genotype (table 2). Moreover, it appears that the acquisition of desiccation tolerance is independent of the environment, as indicated by results obtained in two years and at two locations : during the development of the seed, these differed in terms of heat units available to the plants (fig. 1).

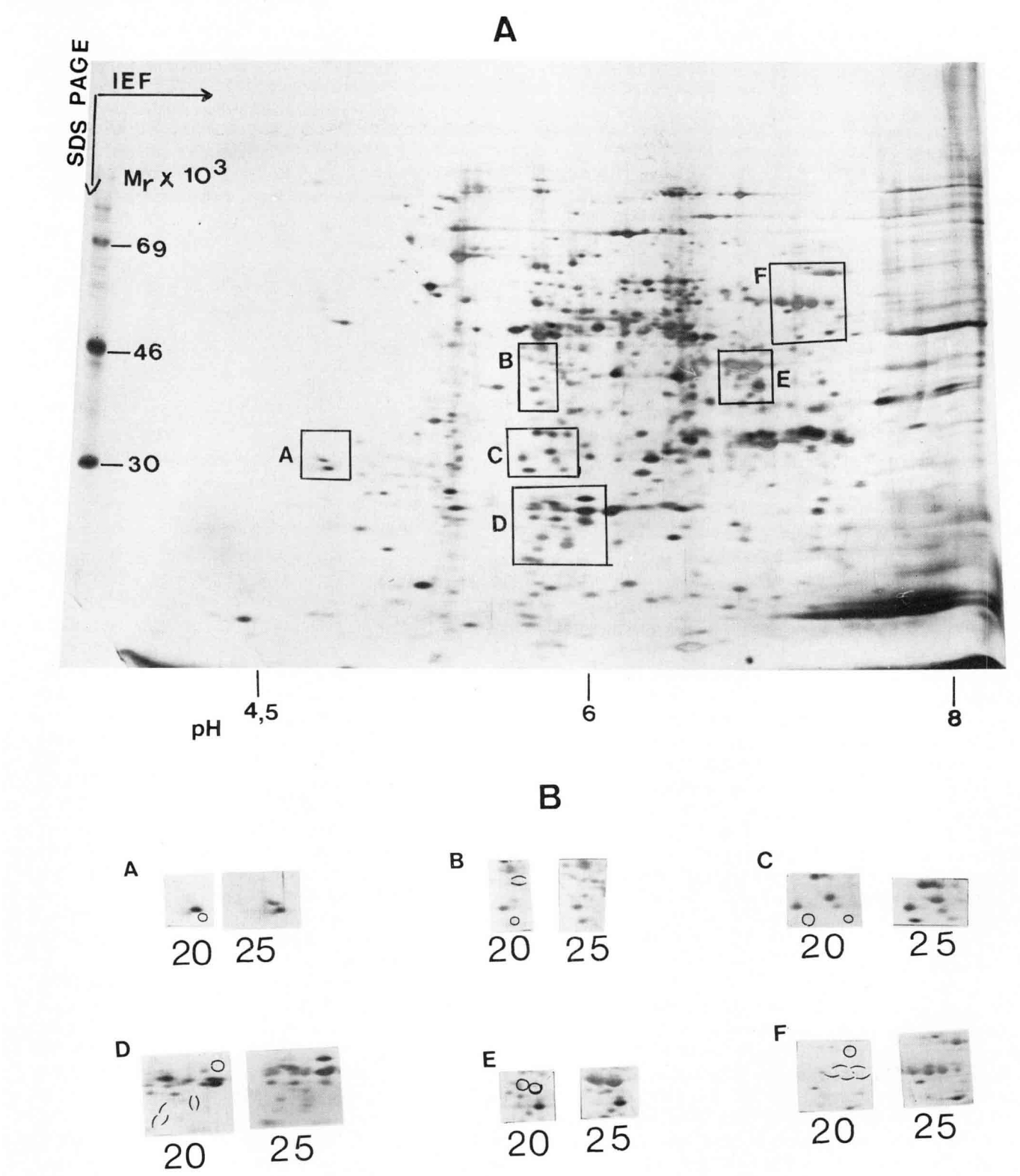

Figure 4

Two-dimensional fractionation (IEF × SDS-Page) of total protein extracted from maize embryos.
4A : 30 DAP dried embryos, desiccation-tolerant stage. The areas (A-E) where the protein patterns change during embryogenesis are indicated. Molecular weights in kilodaltons of 3 standard proteins are indicated on the left, the pH range of the IEF is given on the abscissa.
4B : To compare the protein patterns from the desiccation-intolerant phase to the desiccation-tolerant phase, the relevant areas (marked in fig. 4A) are shown. Proteins from 20 DAP (desiccation-intolerant) and 25 DAP (desiccation-tolerant) are compared. The positions of polypeptides missing in 20 DAP embryos compared to 25 DAP embryos are indicated by open circles.

Electrophorèse bidimensionnelle d'extrait de protéines d'embryons de maïs. A : embryons desséchés à 30 DAP, stade tolérant à la dessiccation. On a encadré les zones (A-E) dans lesquelles le profil protéique change pendant l'embryogenèse. Le poids moléculaire en kilodaltons de trois protéines standard est indiqué sur la gauche de la figure. La gamme du pH du gradient d'électrofocalisation (IEF) est donné en abscisse. B : pour comparer le profil protéique entre la transition de la phase de sensibilité à la phase de tolérance à la dessiccation, on a marqué les aires correspondantes de la figure 4A. Les protéines à 20 DAP et 25 DAP sont comparées. Les positions de polypeptides présents seulement à 25 DAP sont encerclés.

We have also shown that the modifications induced by embryo desiccation on the growth of the seedling after germination become progressively negative, passing from a mature stage to 25 DAP. This indicates that the metabolic and structural processes needed for complete development of the maize embryo are important for full integrity of the seedling after embryo desiccation and germination. It has already been pointed out (CHEN *et al.*, 1968 ; CRÈVECŒUR *et al.*, 1976 ; SARGENT *et al.*, 1981 ; DASGUPTA *et al.*, 1982 ; LALONDE & BEWLEY, 1985) that, at stages preceding the onset of tolerance to desiccation, loss of membrane integrity, structural perturbations as well as disruptions of synthetic events are possibly irreversible changes induced by dessication. The data of table 3 indicate that in maize, post-germination parameters dealing with the normality of the seedling can also be significantly affected by a drying treatment applied at any time between desiccation tolerance and full maturity. These results can indicate that desiccation tolerance and normal behaviour of the seedling after embryo desiccation and germination are physiologically dependent on developmental events partly chronologically separated.

A first attempt to understand the nature of these developmental events was approached by 2-D-electrophoresis of total proteins present in the embryos at stages during development which represent a transition between desiccation intolerance (16 and 20 DAP) and tolerance (25 and 30 DAP).

The observations of newly synthesized proteins at 25 and 30 DAP are in agreement with data from SANCHEZ-MARTINEZ *et al.*, 1986 who reported the expression of an embryonic set (present at 20 DAP) and a maturation set of polypeptides (31-50 DAP) during maize embryogenesis. Nevertheless, comparison between dried and fresh embryos did not reveal any major differences in the protein pattern. Our method of analysing the protein patterns by 2-D-electrophoresis and subsequent silver-staining only reveals proteins present in a certain quantity. Thus, it cannot be excluded that the use of other techniques might reveal subtle drying-induced modifications of the protein pattern. Some of the proteins expressed in later stages of embryogenesis can possibly be accounted for as embryo storage proteins. Whether other proteins can in some way be related to the acquisition of desiccation tolerance of the maize embryo can at present not be decided. The differences observed may open possibilities for approaching the acquisition of desiccation tolerance by the maize embryo at the biochemical level.

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