STUDY ON BIOCHEMICAL MARKERS OF DROUGHT TOLERANCE IN SUNFLOWER *Helianthus annuus L.* BY TWO-DIMENSIONAL ELECTROPHORESIS
First results

Charline GIRARDEAU (1) , André MERRIEN (2) and Alain QUINSAC (1)
CETIOM (Centre Technique Interprofessionnel des Oléagineux Métropolitains)

(1) Laboratoire d'Analyses, Avenue de la Pomme de Pin, 45160 Ardon, France.
(2) Centre de Biologie Appliquée, Rue de Lagny, 77178 Saint Pathus, France.

ABSTRACT

Until now, breeding Sunflower *Helianthus annuus L.* for drought tolerance has been based on morphological or physiological characters. In comparaison with the results obtained in Rapeseed *Brassica napus*, we tried to carry out an experiment on Sunflower under controlled conditions. Compared with a standard of plants irrigated at field capacity, a progressive water stress was applied for 11 days from -0,5 MPa to -2,6 MPa at 2.5 stage (5 leaves). During drought, plants were sampled (roots and leaves) and different protein patterns were investigated by the 2-D PAGE® technique. The first results obtained suggest that the best organs to be analysed were the roots. Different protein patterns are presented and show clearly the occurence of polypeptides synthesis under water stress.

Key words : Sunflower, *Helianthus annuus L.*, drought, biochemical markers, two-dimensional gel electrophoresis

RESUME

Jusqu'à présent, l'amélioration du Tournesol *Helianthus annuus L.* pour la résistance à la sécheresse a été basée sur des critères morphologiques ou physiologiques. A l'image des résultats obtenus chez le colza *Brassica napus*, nous avons tenté de mener une expérience chez le Tournesol cultivé en conditions contrôlées. Parallèlement à un traitement standard irrigué à la capacité au champ, un stress hydrique progressif a été appliqué pendant 11 jours de -0,5 MPa à -2,6 MPa, au stade 2.5 (5 feuilles). Au cours de la sécheresse, des prélèvements de racines et de feuilles ont été effectués et les différents profils protéiques examinés par la technique 2-D PAGE®. Les premiers résultats obtenus suggèrent que les meilleurs organes à analyser sont les racines. Différents profils protéiques sont présentés et montrent clairement l'apparition de la synthèse de polypeptides sous l'effet du stress hydrique.

INTRODUCTION

Indicators actually available to estimate drought tolerance in Sunflower *Helianthus annuus L.* are morphological and physiological. Thus, our purpose was to investigate if under a progressive water stress, we can identify specific biochemical markers.
This research was carried out following previous results obtained in rapeseed *Brassica napus* (Vartanian *et al.*, 1987; Damerval *et al.*, 1988). In those experiments, leaves and roots were analyzed with the 2-D PAGE① technique after 20 days of water shortage :
- In roots, a threshold water deficit induced the formation of short, tuberized, hair deprived roots which were highly tolerant to dessication. Among the 13 polypeptides induced by drought (Mm① ≈ 20 to 34 kD①), two were specifically induced in all the root types.
- In leaves two polypeptides (Mm ≈ 22 kD) appeared in the 2nd, 3rd and 4th leaves.

In comparison with these results, we tried to carry out a similar experiment on Sunflower under controlled conditions.

---

① *Abbreviations* : 2-D PAGE : two-dimensional polyacrylamide gel electrophoresis ;
h : hour ; kD : kilodaltons ; Mm : Molecular mass ;
SDS : Sodium dodecyl sulfate

MATERIALS AND METHODS

Plant culture

Sunflower plants *Helianthus annuus L.* were cultivated in phytotron at a photon flux density of 400 µEinstein.$m^{-2}.s^{-1}$, under a 13 hours daylenght, with a day/night temperature of 20/14°C and 60/90% for relative humidity.
Two plants (A, B) were grown in plastic pot (650 ml) filled with 25% of sand (gravel from Antibes) and 75% of soil (lime-clay), both sterilized.
Three weeks after sowing (2.5 stage), control plants were maintained at field capacity by regular waterings, while drought-stressed plants were progressively submitt to stress for 11 days. The substratum surface was recovered with a polyethylene film in order to prevent evaporation. Thus, water losses was exclusively due to transpiration. For each treatment, two replications were performed.

Measurements and sampling procedure

- The kinetics of the transpiration ($g.h^{-1}.cm^{-2}$) during water stress was determined by weighting pots at the same hour (after 6 hours lighting) and measuring leaf area (cm^2) according to the allometric method (length x width x 0,777).
- Plants A: Water potential of the whole plant was measured with the pressure chamber method (Scholander *et al.*, 1965). The shoot was separated from the root just prior to the measurement .

- Plants B: Organs were separately collected (second pair of leaves and roots). Roots were carefully and quickly washed in cold water and then dried. Three sampling dates was chosen: after 4 days (-0,6 MPa), 7 days (-1,5 MPa) and 11 days (-2,6 MPa) of water stress. Samples from 100 to 200 mg were immediately frozen in liquid nitrogen until the extraction.

Extraction and two-dimensional electrophoresis of proteins

Extraction procedure was performed as described in Zivy (1981) and Damerval *et al.*, (1986). Briefly, total denatured proteins were extracted with direct precipitation in cold acetone (-18°C), trichloracetic acid (10%), 2-mercaptoethanol (0,7%) for 1 hour. After centrifugation, the pellet was washed with 0,7% 2-mercaptoethanol in acetone (-18°C for 1 hour). The vaccum dried pellet was resolubilised in UKS solution (Damerval *et al.*, 1986) (50 to 60 µl/mg) and the protein extracts frozen. 25 or 50 µl (for leaves or roots respectively) were used for electrophoresis.

The 2-D PAGE procedure was modified from O'Farrel (1975) according to Damerval *et al.*, (1987). Isoelectric focusing (IEF) was performed in rod gels of 1,5 mm diameter and 16 cm long. The gel mixture was composed with 25 and 75 % of Pharmalyte pH 5 to 6 and pH 5 to 8 respectively. SDS® electrophoresis was performed with 165 x 200 x 1,5 mm slab gels bound to GelBond PAG films. The equipment was a PROTEAN II cell (Bio-Rad). Molecular mass of polypeptides were estimated using standard proteins (Pharmacia calibration kit) : Phosphorylase b (94 kD), Bovine Serum Albumin (64 kD), Ovalbumin (43 kD), Carbonic Anhydrase (30kD) and Soybean Trypsin Inhibitor (20,1 kD). Silver staining followed the modified procedure described by Granier and De Vienne (1986) and Damerval *et al.*, (1987).

RESULTS AND DISCUSSION

Transpiration and water potential

As shown on *fig.* 1, in water-stressed plants, the transpiration rate dropped regularly until an approximate value of $1,7 \times 10^{-3}$ $g.h^{-1}.cm^{-2}$.

This decline of transpiration, due to stomatal closure, occured simultaneously with a sharp decrease from -0,5 to -2,6 MPa of the water potential (*fig.* 2).

In well-watered plants, the transpiration kept a relatively constant value around 9×10^{-3} $g.h^{-1}.cm^{-2}$ and the water potential at -0,45 MPa.

Comparison of two-dimensional protein patterns

Several experiments were performed for each organ, under various moisture regimes. The dried gels bound to GelBond were examined using a light box. Protein patterns obtained from leaves extracts did not allow us to make a good analysis. The main reason was the abundance of the large subunit of ribulose biphosphate carboxylase-oxygenase (Rubisco) and also many streaks in the gels.

However, the analysis of roots denatured proteins allowed us to clearly detect newly synthesized proteins under water stress conditions (*fig.* 3 a and b). The protein pattern comparison had bear upon six of them (numbered from 1 to 6) for 2 treatments: -0.5 MPa

compared to -2.6 MPa. The 4th, 5th and 6th increase in intensity with water stress (between -1.5 MPa to -2.6 MPa).

Four protein extraction and two-dimensional electrophoresis has been done. For each one, the localization of the 6 reproducible spots is presented on *fig.* 4. Underlined numbers indicate spots position obtained with IEF gels composed with 100 % of pharmalyte pH 3 to 10. Nevertheless, the pH value is the same (ex : for n° 6, pH = 6 to 6,5 in both cases).

CONCLUSION

As water stress intensity increase, we have found qualitative and quantitative changes in the protein patterns of *Helianthus annuus* var. *L.* roots. In particular, newly synthetized proteins and drought affected spots were determined.

Further, thorough studies should be carried out in order to confirm these results and to clarify some points: Did these polypeptides occur only under water stress? Are they responsible for specific adaptation to drought? Do they disappear upon rehydratation ?

Following researches will try to bring up a point about such questions. Then, it will be possible to look for specific proteins in different genotypes, including wild species. Characterization of those polypeptides could also be helpful to understand the mecanism involved in drought tolerance. In rapeseed, (Vartanian, pers. comm.), water-stressed induced polypeptides had been identified as proteases inhibitors. If such a conclusion could raise up from our next results, there will be evidence that under water stress, the adaptation to drought for Sunflower could came from synthesis of specific proteins able to prevent proteins breakdown and leaf senescence.

Acknowledgments : We wish to thank N. Vartanian and C. Damerval (INRA / CNRS, Gif-sur-Yvette) for their advices and suggestions.

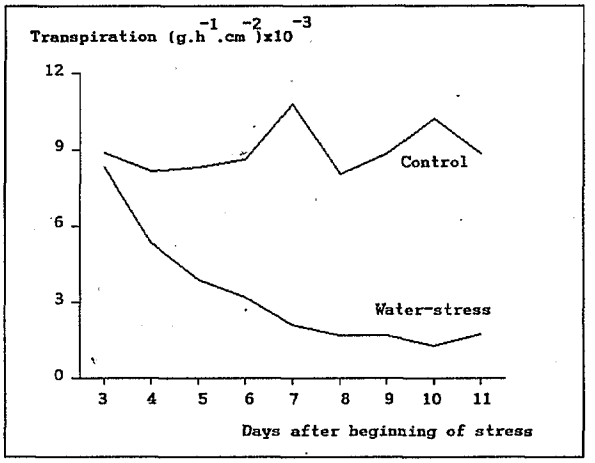

Figure 1 : Kinetics of transpiration in water-stress plants compared to the control.

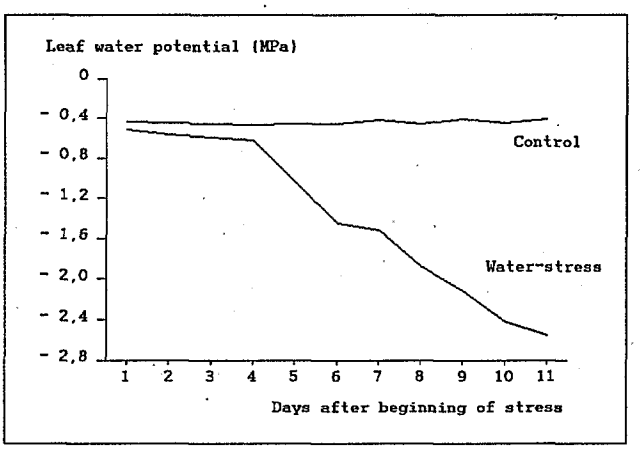

Figure 2: Evolution of leaf water potential in water-stress plants compared to the control.

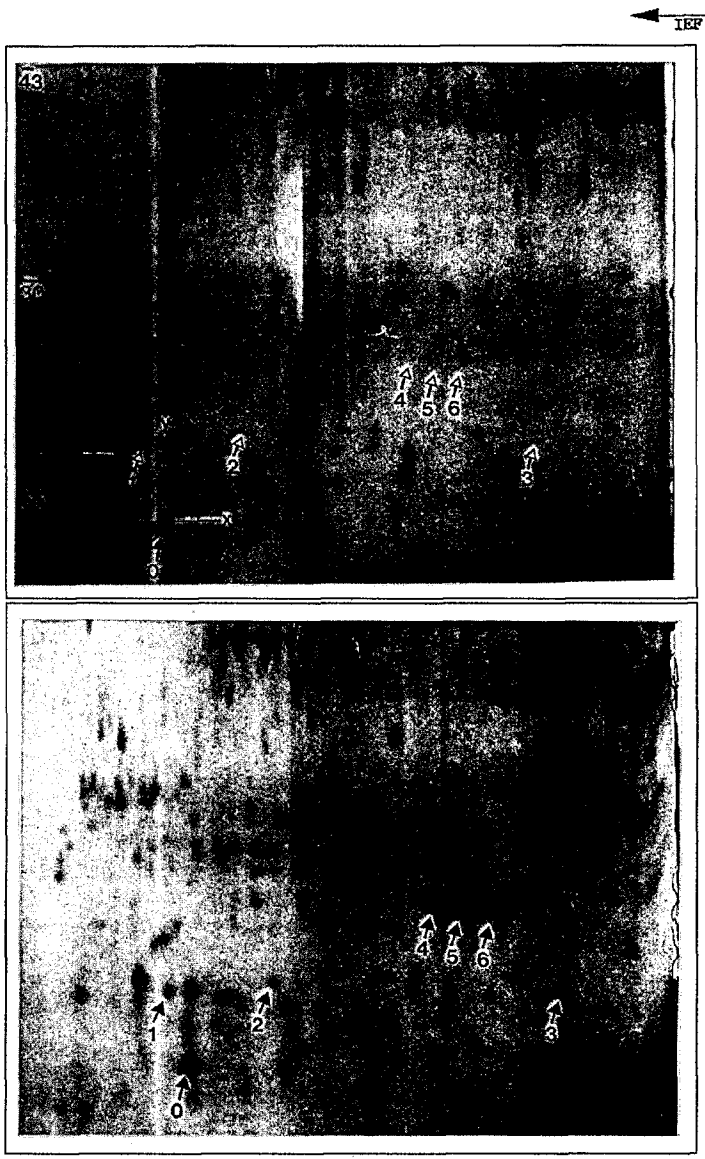

Figure 3 : Detailed portion of two-dimensional roots gels under various moisture regimes
a : well watered roots, -0,45 MPa
b : water-stressed roots, -2,6 MPa
Arrows indicate newly synthetized polypeptides. The scale on the left of Fig. 3a indicates molecular weight standards in kD.

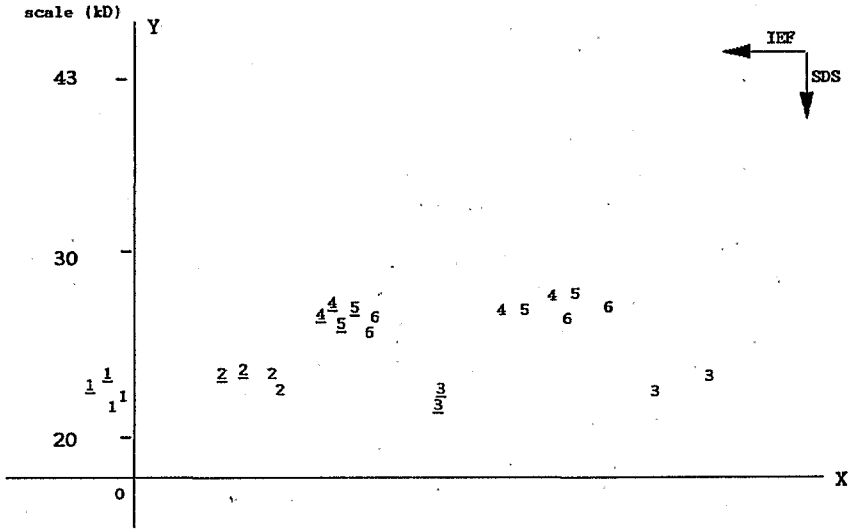

Figure 4 : Position of the 6 spots (1 to 6) obtained through the 4 replications in a (B,Y) plane. The origine is the spot numbered "0" in figure 3.

REFERENCES

DAMERVAL C., De VIENNE D., ZIVY M., THIELLEMENT H., 1986 : Technical improvments in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. Electrophoresis 7 : 52-54.

c66EDAMERVAL C., Le GUILLOUB M., LAISONNEAU J., De VIENNE D., 1987 : A simpli- fication of the Heukeshoven and Dernick's silver staining of proteins. Electrophoresis 8 : 158-159.

DAMERVAL C., VARTANIAN N., De VIENNE D., 1988 : Differential Two-Dimensional Protein Patterns as Related to Tissue Specificity and Water Conditions in *Brassica napus* var. *oleifera* Root Systems. Plant Physiol. 86 : 1304-1309.

GRANIER F., De VIENNE D., 1988 : Silver Staining of Proteins : Standardized Procedure for Two-Dimensional Gels ound to Polyester Sheets. Anal. iochem. 155 : 45-50.

O'FARREL P.H., 1975 : High resolution two-dimensional electrophoresis of proteins. J. iol. Chem. 250 : 4007-4021.

SCHOLANDER P.F., HAMMEL H.T., RADSTREET E.D. and HEMMINGSEN E.A., 1965 : Sap Pressure in vascular plants. Science, 148 : 339-346.

VARTANIAN N., DAMERVAL C., De VIENNE D., 1987 : Drought-Induced Changes in Protein Patterns of *Brassica napus* var. *oleifera* Roots. Plant Physiol. 84 : 989-992.

ZIVY M., 1981: Influence des ampholytes sur la révélation des protéines au nitrate d'argent . Recent Progresses in Two-Dimensional Electrophoresis. Press. Univ. Nancy, pp. 69-72.