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In vitro selection and characterization of drought-tolerant plants of durum wheat (*Triticum durum* Desf)

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Summary — Somaclonal variation has been used as a source of variability to improve the drought tolerance of durum wheat (*Triticum durum* Desf). Of the 6 varieties currently grown in Morocco, 3 were selected for their ability to produce calli and regenerate plants *in vitro*. Using polyethylene glycol 10 000 in the *in vitro* culture medium, low external water potentials were applied during callus proliferation and plant regeneration phases to select plantlets that were tolerant to drought. Out of the 30 plants that survived the stress, 13 exhibited improved tolerance to drought compared with unselected controls, using chlorophyll fluorescence and electrolyte leakage of leaves subjected to water stress as criteria. Applying osmotic stress during the regeneration phase seemed to be the most efficient.

durum wheat / *in vitro* selection / drought tolerance / PEG

Résumé — Sélection *in vitro* et caractérisation de plantes de blé dur (*Triticum durum* Desf) tolérantes à la sécheresse. *La variation somaclonale a été utilisée comme source de variation génétique pour l'amélioration de la tolérance à la sécheresse chez le blé dur (*Triticum durum *Desf). Parmi 6 variétés largement cultivées au Maroc, 3 ont été choisies sur leur capacité à développer des cals et à régénérer des plantes* in vitro. *L'addition de polyéthylène glycol 10 000 aux milieux de culture a été utilisée pour réduire le potentiel hydrique externe, pendant la croissance des cals ou durant la phase de régénération, et sélectionner des plantules tolérantes à la sécheresse. Sur les 30 plantules régénérées à partir des cals survivants, 13 montrent une amélioration de la tolérance par rapport aux témoins, sur la base des mesures de la fluorescence de la chlorophylle et des pertes d'électrolytes effectuées sur les feuilles en présence d'un stress hydrique. La sélection sur milieu de régénération semble la plus efficace.*

blé dur / sélection* in vitro / *tolérance à la séchesse / PEG

INTRODUCTION

Drought affects approximately 40% of the cultivated land throughout the world, reducing markedly crop yield (Oertli, 1983). In wheat, water stress influences all phases of development from germination up to grain filling, through vegetative and reproductive growth. Owing to the fact that this species, which is of great economic importance, has to endure drought conditions in numerous countries where it is cultivated, its future production will largely depend on studies devoted to a better understanding of this stress.

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Improved field techniques, particularly the use of irrigation, resulted in a lessening of the detrimental effects of water deficit. When irrigation is unfeasible, however, genetic improvement probably remains a choice method for obtaining plant material capable of satisfactory production under drought conditions.

The aim of our study was to exploit *in vitro* techniques for production, selection, and regeneration of durum wheat somaclonal mutants that are tolerant to drought. The stress was induced by adding high molecular weight polyethylene glycol (PEG) to the culture medium. *In vitro* selection using PEG as an osmotic agent allowed the regeneration of plant lines tolerant to drought in various species including tomato, rice and tobacco (Heyser and Nabors, 1979; Bressan *et al*, 1981, 1982; Kishor and Reddy, 1984; Sumaryati *et al*, 1992). Chlorophyll fluorescence and electrolyte leakage served as indicators of drought tolerance (Havaux *et al*, 1988; Vasquez-Tello *et al*, 1990).

MATERIALS AND METHODS

Plant material

Six varieties (Kypérounda (2777), Sebou (1715), Selbéra (272), E21, E15 and Massa (1728)) were provided by the INRA from Morocco and were tested for their *in vitro* potentialities. The first 3 were selected for the present study because they were able to produce calli and plantlets at high rates.

Mature embryos were used as explants. The caryopses were surface sterilized first using ethanol (94%) for 10 s followed by formaldehyde (0.75%) for 40 min with continuous shaking, and $Ca(ClO)_2$ (5%) for 20 min. They were then rinsed 3 times with sterile water before being incubated in Petri dishes on Whatman paper imbibed with sterile water.

After a 24 h pre-germination period under a 12 h light/12 h dark cycle at 25°/20°C (day/night), the embryos were excised and transferred, scutellar side up, to the callogenesis medium.

Culture media

Two basic culture media were used: MS (Murashige and Skoog, 1962) and LS (Linsmaier and Skoog, 1965). Growth factors added to induce callogenesis and support callus development were 0.495 mg/l 2,4-D–2,4-dichlorophenoxyacetic acid, 1 mg/l naphthaleneacetic acid (NAA) and 1 mg/l benzylaminopurine (BAP). Two regeneration media were used (R1 and R2); R1 (for Kypérounda and Sebou) was based on the LS mineral elements at half-concentration for macronutrients, with 1 mg/l BAP, 1 mg/l NAA and 1.5% sucrose, and R2 (for Selbéra) contained the MS elements at half-concentration for macroélements, 10.75 mg/l kinetin, 1.752 mg/l indoleacetic acid (IAA) and 3% sucrose. After regeneration, the plantlets were transferred to a regeneration medium with 1 mg/l IAA for rooting.

Polyethyleneglycol 10 000 (PEG), which is a non-toxic hydrosoluble polymer, is used in the *in vitro* culture medium for drought-resistance selection. PEG simulates water stress by reducing the free water in the extracellular medium and the water available to the cells.

Evaluation of drought tolerance

After regeneration, rooted plantlets were washed, planted in pots and cultivated in a growth room with a day length of 12 h; the temperature was 30°C during the day and 25°C during the night. The light intensity was about 200 $\mu E \cdot m^{-2} \cdot s^{-1}$ and the relative humidity 90%. The plants received modified Hoagland solution (Gulick and Dvorak, 1987) every 10 d.

Chlorophyll fluorescence and electrolyte leakage, which are usually considered as appropriate tests for drought-tolerance evaluation (Havaux and Lannoye, 1985; Havaux *et al*, 1988; Martin *et al*, 1987, Vasquez-Tello *et al*, 1990), were used to estimate the response of the regenerated plants to water stress and to compare these plants to unselected genotypes. The stress was applied to the youngest completely unfolded leaf of plantlets with 3–4 leaves by placing leaf segments on a filter paper, in a Petri dish, for 6 h, in the dark and at 25°C.

The technique used for measuring the chlorophyll fluorescence has been described by Havaux and Lannoye (1985) and Havaux *et al* (1988). The portable Hansatech fluorimeter gives an estimation of the efficiency of primary photochemical processes of PSII based on the ratio between variable and maximum fluorescence (F_v/F_m). Three readings were recorded for each sample; the mean value was then compared to the ratio obtained for unstressed leaves.

To measure the electrolyte leakage, the segments of the young unfolded leaf were divided into 3 batches in order to get 3 replications. The fragments were thoroughly washed to remove all electrolytes from their surface and from the wounded parts (Blum and Ebercon, 1981). After desiccation of the samples, the proportion of electrolytes released in the water was estimated by conductimetry, according to Martin *et al* (1987).

RESULTS

Determination of stress conditions for selection

PEG concentration and stress duration must be sufficient to prevent the growth of original cells but to allow the proliferation of tolerant cells. Three PEG concentrations (15, 20 and 25%) were tested and compared with the standard culture media. Thirty 36-day-old calli were grown for each PEG concentration and variety. The weight of the calli was determined before treatment and at each monthly transfer for 6 months. In order to reduce the effect of initial weight on the growth rate, the inoculated calli had similar weights for all varieties. The growth of the calli as a function of duration of the PEG treatment is illustrated in figure 1. For the 3 varieties, callus growth was significantly reduced by the presence of PEG from the first month onward. The stress effect increased with PEG concentration. The growth was almost stopped after 6 months for the highest PEG concentrations, which appeared to be the most suitable for selection.

Based on these preliminary observations, we elaborated 3 different stress procedures for drought-resistance selection: (i) the calli were cultured on a solid medium with PEG (25%) for 6 months; (ii) the selection was performed during the regeneration phase by growing calli for 2 months on the R1 or R2 media containing PEG 25%; and (iii) a shorter selection period (30 d) was used for growing calli in a liquid medium (LS) with PEG 35% before regeneration.

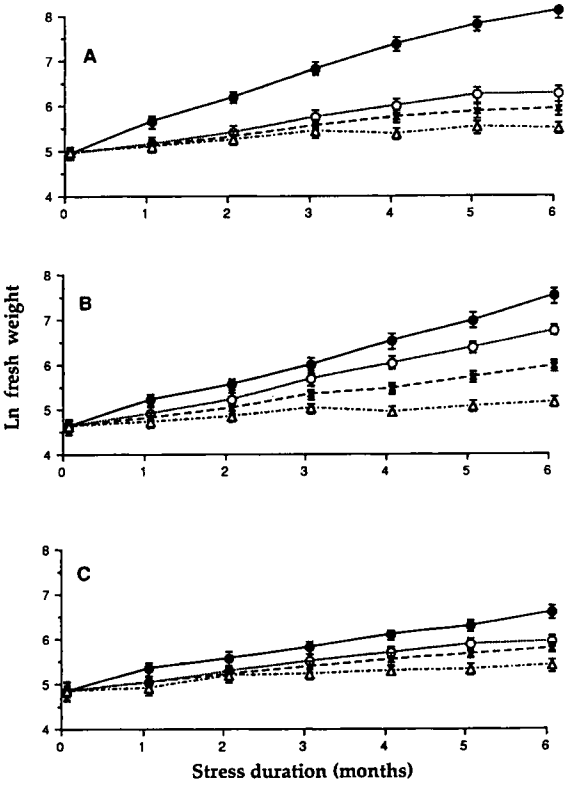

Fig 1. The effect of PEG on callus growth as a function of the stress duration in (**A**) Kypérounda, (**B**) Sebou, and (**C**) Selbéra. PEG concentrations: ● = 0%, ○ = 15%, x = 20%, and △ = 25%. Data are given as mean ± SD.

In vitro *selection and plant regeneration*

When grown under stress conditions, most calli became necrotic and many of them died, but some cell groups remained able to grow and to regenerate plantlets. While untreated calli often gave 3–4 plantlets, only one appeared on the most stressed calli.

For the first selection procedure, 3 500 embryos from each of the 3 varieties were cultured on callus-inducing medium for 1 month before being transferred to the selective medium for 6 months. At the end of the selection pressure, the non-necrotic calli were transferred to the regeneration medium where 2 Kypérounda, 5 Sebou and 4 Selbéra plants were recovered after 2 months. These plants are referred to as RoS.

For selection applied at the regeneration stage, 1 000 embryos per variety were cultured during 1 month on the induction medium before being transferred to the regeneration medium containing PEG. After 2 months, 3 Kypérounda, 5 Sebou and 5 Selbéra plants were obtained. These plants are referred to as RoR.

For the third selection method, 1 000 embryos per variety were cultured for 1 month on the induction solid medium before being transferred for 1 month further to the liquid medium containing PEG. The calli were then kept for 2 months on the regeneration medium where 2 Kypérounda and 3 Sebou plants were produced. These plants are referred to as RoL.

The 30 plants regenerated on selective media were grown up to maturity.

Estimation of drought tolerance of regenerated plants

Chlorophyll fluorescence and electrolyte leakage were measured for dehydrated leaf samples of the plants regenerated from the cellular lines selected for their drought tolerance. The results are shown in 3 separate tables for the plants issued from each of the 3 Moroccan parental lines (tables I, II, III). They indicate that 13 individuals (5 Kypérounda, 5 Sebou and 3 Selbéra) out of the 30 plants tested exhibited an improved tolerance in comparison to the parental genotypes. The data from chlorophyll fluorescence and electrolyte leakage were consistent.

Table I. Chlorophyll fluorescence and electrolyte leakage in Kypérounda plants selected for their resistance to drought, as influenced by a 6-h dehydration stress applied to leaf samples.

Plant	F_v/F_m *(% of non-stressed control)*	*Electrolyte leakage (% of total)*
Unselected plant from the parent genotype	48.74 ± 5.21	70.88 ± 7.91
RoS1	50.00 ± 4.32	71.14 ± 5.69
RoS2	59.00 ± 3.25*	61.89 ± 6.42*
RoR1	49.00 ± 2.56	70.00 ± 5.89
RoR2	47.00 ± 3.89	71.00 ± 8.12
RoR3	80.00 ± 4.58*	59.2 ± 5.99*
RoR4	87.00 ± 6.12*	62.19 ± 4.78*
RoL1	67.00 ± 7.15*	61.68 ± 6.89*
RoL2	61.00 ± 6.56*	64.01 ± 5.96*

Data are given as mean ± SD; *plants that are statistically different from unselected plants using Fisher's test at a 95% probability level.

Table II. Chlorophyll fluorescence and electrolyte leakage in Sebou plants selected for their resistance to drought, as influenced by a 6-h dehydration stress applied to leaf samples.

Plant	F_v/F_m *(% of non-stressed control)*	*Electrolyte leakage (% of total)*
Unselected plant from the parent genotype	59.95 ± 3.89	79.21 ± 6.14
RoS1	47.75 ± 5.78	78.49 ± 5.89
RoS2	62.25 ± 6.14	81.89 ± 7.45
RoS3	50.75 ± 5.66	80.51 ± 8.23
RoS4	80.37 ± 6.97*	60.00 ± 7.02*
RoS5	51.00 ± 4.13	79.15 ± 6.15
RoR1	76.87 ± 4.68*	68.32 ± 5.78*
RoR2	68.12 ± 7.15*	68.06 ± 7.03*
RoR3	72.13 ± 5.57*	63.59 ± 4.86*
RoR4	52.12 ± 5.36	79.56 ± 5.15
RoR5	54.50 ± 4.65	78.26 ± 4.79
RoL1	49.50 ± 3.26	81.09 ± 8.06
RoL2	56.15 ± 2.98	72.98 ± 4.64
RoL3	56.15 ± 4.65*	60.07 ± 5.66*

Data are given as mean ± SD; *plants that are statistically different from unselected plants using Fisher's test at a 95% probability level.

Table III. Chlorophyll fluorescence and electrolyte leakage in Selbéra plants selected for their resistance to drought, as influenced by a 6-h dehydration stress applied to leaf samples.

Plant	F_v/F_m *(% of non-stressed control)*	*Electrolyte leakage (% of total)*
Unselected plant from the parent genotype	62.24 ± 6.11	74.25 ± 8.17
RoS1	62.75 ± 2.44	76.25 ± 5.29
RoS2	61.87 ± 2.25	78.68 ± 4.05
RoS3	63.00 ± 5.33	67.95 ± 7.11
RoS4	71.37 ± 6.01*	60.57 ± 4.45*
RoR1	60.00 ± 4.13	76.50 ± 4.66
RoR2	61.87 ± 4.11	74.40 ± 6.30
RoR3	63.00 ± 3.14	73.25 ± 7.33
RoR4	72.50 ± 3.99*	66.00 ± 5.44*
RoR5	70.00 ± 4.09*	64.95 ± 6.15*

Data are given as mean ± SD; * plants that are statistically different from unselected plants using Fisher's test at a 95% probability level.

CONCLUSIONS

Using chlorophyll fluorescence and electrolyte leakage as indicator tests, improved drought-tolerant plants were regenerated after selection on culture media containing PEG. Since these physiological tests, especially electrolyte leakage, are sometimes considered to provide a measure of membrane integrity (Martin et al, 1987; Vasquez-Tello et al, 1990), it could be suggested that the selected character is related to a modification of the properties of the cell membranes that are able to keep their integrity in the presence of water stress. The stability and the heredity of this selected character remain, however, to be established since transient, not heritable, physiological adaptations of cells grown under stress conditions may occur (Demarly, 1986). This phenomenon could also account for the plants regenerated from selected calli that did not differ significantly from the original genotypes. The heterogeneity of the plant populations frequently recovered after *in vitro* selection (Dix, 1977) could also be due to the persistence of some original non-mutated cells in the selected calli (Demarly, 1986).

According to Nabors *et al* (1980), the presence of a selective agent in the regeneration medium can increase the probability of recovering tolerant plants. In this way, NaCl-tolerant plants have been obtained in *Oryza sativa* (Li and Heszki, 1986) and *Kickxia ramosissma* (Mathur *et al*, 1980). In our experiments, the 3 selection procedures have been successful, but it seems that the application of the osmotic stress during the regeneration phase was the most efficient: 7 plants showing an improved tolerance were obtained with such a treatment and only 3 for each of the other methods.

The progeny of the selected plants is currently under study to establish whether the selected character is stable and heritable.

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