Artikel: Use of fast chlorophyll : a fluorescence technique in detecting drought and salinity tolerant chickpea (Cicer arietinum L.) varieties

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DOI: https://doi.org/10.5169/seals-740432

Archs Sci. Genève	Vol. 56	Fasc. 2	pp. 79-93	Novembre 2003

USE OF FAST CHLOROPHYLL A FLUORESCENCE TECHNIQUE IN DETECTING DROUGHT AND SALINITY TOLERANT CHICKPEA (*CICER ARIETINUM* L.) VARIETIES

BY

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(Ms. reçu le 24.11.2003, accepté le 28.11.2003)

ABSTRACT

Use of fast chlorophyll a fluorescence technique in detecting drought and salinity tolerant chickpea (*Cicer arietinum* L) varieties. - Chlorophyll a fluorescence analysis is recognized to be a useful tool to quickly and easily detect influence of many different stress types on plants as drought, heat, cold, atmospheric, soil or groundwater contaminants. The present study was undertaken in order to check if these methods could also be useful for comparing chickpea (*Cicer arietinum*) varieties for their ability to tolerate drought and salinity stress. The results show that among the 9 varieties tested there exist important differences between the varieties to withstand drought or salt stress conditions for a certain time and to recover from it. Some varieties recover to about the level of the unstressed controls after drought conditions. However no recovery was detected from salt stress.

The chosen method appears to be a useful tool in comparison of differential stress tolerance in plants.

Key-words: Chlorophyll fluorescence, stress, drought, salinity, JIP-test, Performance Index.

INTRODUCTION

Chickpea, *Cicer arietinum* L. constitutes the 3rd important pulse crop worldwide (SINGH & SAXENA, 1999 p. 1). It is a member of the Fabaceae family and mainly grown in tropical, sub-tropical and temperate regions of the world as a rain fed, cool weather crop or as a dry crop in semi-arid regions, favoring the optimum growth conditions of 21-29 °C during day and 18-26 °C at night (MUEHLBAUER *et al.*, 1997). Since chickpea forms a deep reaching taproot able to absorb ground water it is capable to withstand rather arid conditions, and thus can produce good yields in drier regions. India is the most important chickpea producer: 6'560'000 ha and 5'537'000 t in 1994 (SINGH & SAXENA 1999, p. 2f). Poor soil, use of unimproved varieties and low rainfall is predominant (OPLINGER *et al.*, 1990) for culturing this crop. Because of the minimal dependence on monetary inputs such as N and P containing fertilizers, irrigation and agrochemicals, chickpea is known to confer and contribute to sustainability in cropping systems.

In the sustainable development of agriculture it is important to increase the productivity of crops with available resources. Hence, the identification of the crops or the varieties that can withstand a wide range of environmental conditions, especially when

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unfavorable ones create stress to plants, becomes a worldwide requirement. Therefore it is important to know the physiological and molecular mechanisms by which water deficit and salinity inhibits growth and development of plants in order to select more tolerant plants lines as well as for genetic engineering to produce more resistant lines for salinity and drought stress.

Drought stress has several effects on plant growth of which limitation in leaf expansion is most important. As a result of drought stress the stomata close to reduce the evaporation from the existing leaf area and thus reducing gas exchange simultaneously what leads to CO_2 depletion. As well water deficiency enhances the synthesis of abscisic acid (ABA) which is accumulated in the chloroplast by changing the pH which is a crucial factor in enzyme activity and causes changes of photosynthesis reactions. The dehydration of mesophyll cells inhibits photosynthesis and as stress becomes severe, water use efficiency decreases and the inhibition of mesophyll metabolism becomes stronger. The other evidence suggests that the Mg^{2+} concentration in chloroplasts influences photosynthesis during water stress through its role in coupling electron transport to ATP production (McKersie & Leshem, 1994, Taiz & Zeiger, 1998), thylakoid fusion and stacking.

Due to the accumulation of excess amounts of anions like $C1^-$, SO_4^{2-}, CO_3^{2-}, HCO_3^- and cations of Na^+, K^+, Mg^{2+} and Ca^{2+} (Greenway & Munns, 1990), the soil salinity is increased. Though the most occurring salinity problem is created by the accumulation of NaC1 in soils (Misra *et al.*, 1995). The first plant responses to salt stress are a reduction in leaf growth rate with associated reductions in leaf area available for photosynthesis. Subsequently, the excessive accumulation of salts can lead to death of tissues, organs and whole plants (Munns 1993). Photosynthesis is inhibited when high concentrations of Na^+ and $C1^-$ accumulate in the chloroplasts. Since photosynthetic electron transport appears relatively insensitive to salts, either carbon metabolism or photophosphorylation may be affected (Cramer *et al.*, 1985). High concentrations of NaC1 in the soil affect especially the PSII photochemical efficiency, charge separation of primary charge pairs in PSII and pigment protein complexes of thylakoid membrane (Misra *et al.*, 1999).

In photosynthesis, photosystem II (PSII) has been considered as rather sensitive to stress conditions. Therefore PSII analysis becomes more reliable than any other techniques in the studies analyzing stress effects on plants. Chlorophyll a (Chl a) fluorescence, the dissipated energy emitted by PSII after exciting Chl a molecules by light energy, can be considered as very useful and non-invasive tool for the investigation of stress effects on PSII as well as its structure and function (Strasser *et al.*, 1996; Strasser *et al.*, 2000; Tsimilli-Michael & Strasser, 2001).

The fluorescence transient, also known as Kautsky transient consists of a polyphasic rising curve with a slower decline to a stready state. The shape of this curve is known as OJIP fluorescence transient. The rise of the curve from the initial level O to its maximum peak P reflects the accumulation of the reduced form of the primary electron acceptor quinone (Q_B^{2-}) due to the PSII activity. Since PSII is very sensitive to many environmental changes, the shape of the fluorescence transient curve was also found to depend on different environmental factors such as temperature fluctuations, drought, salinity, light intensity, chemical influences, atmospheric CO_2 (Tsmilli-Michael & Strasser,

2001) as well as during leaf development (SRIVASTAVA *et al.*, 1999). Since that, the fluorescence transient measurements have been used to analyze the stress effects on plants using a portable fluorimeter PEA (Plant Efficiency Analyser, built by Hansatech Ltd., King's Lynn, GB) by which the fast fluorescence kinetics can be measured *in vivo* and *in situ* with a 10μs time resolution and a measuring time of one second to many minutes (STRASSER *et al.*, 2001). Once a leaf has been kept in dark for some time (45 to 60 minutes), all the reaction centers (RC) are fully open and their associated quinones fully oxidized, and therefore only a small part of energy is dissipated when illumination starts (= initial fluorescence F_0). When all the reaction centers are closed and their quinones fully reduced, fluorescence (= energy dissipation) passes through a maximum value (F_M).

The advantage of the fast fluorescence measuring technique is that it is easy, non invasive, quick and once the data have been obtained, they can be converted to quantitative and qualitative parameters that can be related to the photochemistry of PSII (STRASSER *et al.*, 2001) (Table 1). Therefore, this can be considered as a very convenient method for analyzing drought and salt resistant ones among nine varieties of chickpea.

TABLE 1: Summary of the JIP test formula (STRASSER *et al.*, 2001)

Quantum Efficiencies or Yields	
φ_{Po} or $TR_0/ABS = 1 - (F_0/F_M) = F_V/F_M$	
φ_{Eo} or $ET_0/ABS = [1 - (F_0/F_M)] * \Psi_0$	
Ψ_0 or $ET_0/TR_0 = 1 - V_J$	
Specific fluxes or Specific activities	
$ABS/RC = M_0 * (1/V_J) * (1/\varphi_{Po})$	
$TR_0/RC = M_0 * (1/V_J)$	
$ET_0/RC = M_0 * (1/V_J) * \Psi_0$	
$DI_0/RC = (ABS/RC) - (TR_0/RC)$	
Phenomenological fluxes or phenomenological activities	
$ABS/CS_0 = F_0$	
$TR_0/CS_0 = \varphi_{Po} * (ABS/CS_0)$	
$ET0/CS_0 = \varphi_{Po} * \Psi_0 * (ABS/CS_0)$	
$RC/CS_0 = \varphi_{Po} * (V_J/M_0) * F_0$	

For F_0 the fluorescence emission at 50 μs after light onset has been taken.

Abbreviations used:

ABS	absorption	= proportional to [antenna chlorophyll]
RC	reaction center	= proportional to [RC-chlorophyll]
TR	trapping	= transfer of absorbed energy to the RC
ET	electron transport	= electrons moving further than Q_A^-
DI	dissipation	
CS	cross section (= leaf part measured)	
M_0	initial slope = $4 * (F_{300\ \mu sec} - F_{50\ \mu s}) / F_V$	
F_0, FV, FM	initial, variable (= maximum — initial) and maximum fluorescence	
V_J	$(F_{2\ ms} - F_{50\ \mu s}) / F_V$	
Index 0	Value at time = 0	
γ	Chl_{RC} / Chl_{total}	
Chl total	$Chl_{RC} + Chl_{Antenna}$	
RC / ABS	$\sim Chl_{RC} / Chl_{Antenna} = \gamma / (1 - \gamma)$	

The main objective of this study is the identification of chickpea varieties that are more resistant to drought and salinity stress. Since drought and salinization is a world-wide problem with vast changes in the environment, most of the cultivated lands tend to convert to unproductive bare lands. Increasing salinity of irrigation water inhibits the agricultural productivity in many semi-arid and arid regions of the world. Therefore, the identification of crops that can withstand harsh conditions becomes important to provide more food for increasing population.

Materials and Methods

Plant material:

Following chickpea varieties have been used,

(1) ICCV-10	(4) C-235	(7) PUSA-256
(2) ICCV-1	(5) Pant G-114	(8) PUSA-1053
(3) ICCV-96029	(6) Annigeri	(9) PUSA-362

The cultivars were selected of our Indian project partners (Prof. Parda Saradhi, Dr. Sharmila Peddisetti and coworkers University of Delhi) and they also provided the seed.

The plants were grown in black plastic pots containing 4 liters of commercial peat soil in the greenhouse at day temperature of 25 °C with the plant density of 3 plants per pot and 10 replicates per variety. Throughout the whole experiment the plants were grown under long-day conditions (16 h light, 8 h dark) by giving additional light if needed (OSRAM HQIT 400W lamps were used). Three weeks old plants were subjected to the treatments drought and high salt conditions.

(a) Effects of drought:

The plants were prepared for three treatments as control (C), drought stress (D) and recovery after subjected to drought stress (DR). While the control plants were watered, the other two were subjected to drought stress without adding water during 3 weeks. From day 22 on the pots prepared for detecting recovery were rewatered like the control pots. Each treatment had for minimum three replicates with three plants per pot. Fluorescence measurements were taken at the day when watering stopped and thereafter in 2 to 5 days intervals. The experiment was stopped after 34 days when most stressed plants showed heavy drought symptoms (70 – 100% dead plant material.). During the whole experiment about 150 leaflets were measured for each variety.

(b) Effects of salt:

Plants were prepared for salt treatments similar as for drought stress including three treatments, control (C), stress (S) and stress recovery (SR). In this experiment 2 pots with 3 plants per treatment were used for each variety. The plants were treated with salt by pouring daily 500 to 750 ml of 250 mM NaCl solution onto the pots until they appeared severely affected (symptoms were grayish leaflets). Rewatering was started for the pots reserved to observe recovery at day 14. Fluorescence measurements were taken from all

the plants before the treatments started and thereafter every one to four days. The experiment was terminated 17 days after salt treatment started when most stressed and recovering plants were 80 to 100% necrotic. During the whole experiment about 180 leaflets per variety were measured.

Fluorescence measurements:

Fluorescence measurements were taken from detached 1 h dark-adapted leaf samples using the HandyPEA (Plant Efficiency Analyser, Hansatech Ltd, UK) fluorimeter. All the measurements were taken at room temperature. Dark-adapted samples were illuminated homogeneously over an area of 4 mm diameter with three light emitting diodes (LED's) and the thus induced fluorescence was recorded in digital form. Digitalization starts with a 10 µs resolution and increases with time. A total measuring time of 1 second was used throughout the experiments.

From these measurements, the following biophysical parameters were calculated (Table 1),

(a) the specific energy fluxes or specific activities
(b) the flux ratios or yields
(c) phenomenological energy fluxes per excited leaf cross section

The initial fluorescence measurement (F_0 was measured at 50 µs), the maximum fluorescence value where all the reaction centers are physiologically closed (F_M) and the variable fluorescence ($F_V = F_M - F_0$) were used to describe the fluorescence kinetics.

The flux ratios that relate to the yield have been calculated as reported (STRASSER *et al.* 2000).

In addition a so-called Performance Index PI was calculated as,

$$PI = \{\gamma / (1 - \gamma)\} * \{\varphi_{Po}/(1-\varphi_{Po})\} * \{\Psi_0/(1-\Psi_0)\}$$

where γ = fraction of RC chlorophyll / total chlorophyll

Substituting these biophysical parameters by the experimental parameters, we get

$$PI = \frac{1 - (F_0/F_M)}{M_0/V_j} * \frac{(F_M - F_0)}{F_0} * \frac{(1- V_j)}{V_j}$$

where $M_0 = 4*(F_{300\mu s} - F_{50\mu s})/(F_M - F_{50\mu s})$ = initial slope per 1 ms

The PI is an expression which combines the three main categories of forces driving photosynthesis (Fig. 1):

- Concentration of reaction centers of PSII.
- Performance of the light reaction.
- Performance of the dark reaction.

Therefore the PI can be used as an indicator for the potential biomass production as has been observed in several cases (CALANTZIS, 2002).

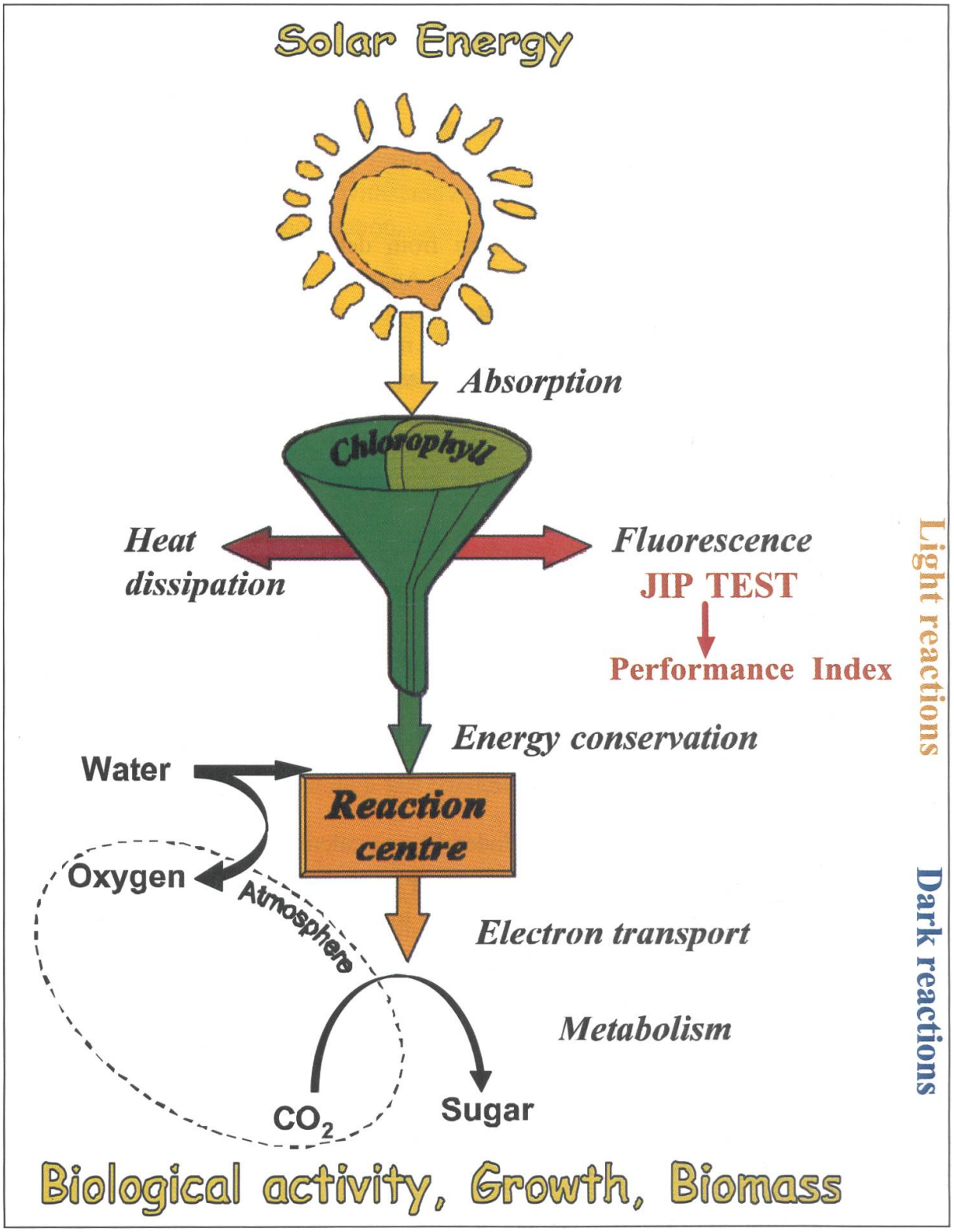

FIG. 1. Schematic representation of the major important events during photosynthesis.

Results and Discussion

The fluorescence measurements have been used to calculate the quantum efficiencies (φ_{Po}, φ_{Eo} and Ψ_0), specific fluxes (ABS/RC, ET_0/RC) and some phenomenological parameters (ABS/CS_0, ET_0/CS_0, RC/CS_0), and were compared to the non-treated control samples.

(a) Drought Effects

After exposing all nine varieties to drought stress condition, no significant changes have been observed in fluorescence parameters until day 12 after watering stopped. At this time no visible symptoms could be observed on any plant. After this time the effects on the absorption per reaction center showed a considerable deviation from the control samples (Fig. 2 for var. 1 (ICCV-10) as an example) and appeared to be a first symptom of drought. The other varieties all behave similarly. In order to simplify data representation the data were pooled to give 3 periods.

- **Before drought (B):** measurements from day 0 to 12 (all controls)
- **Drought (D):** measurements of day 16 to 22 included watered controls and unwatered drought stressed plants.
- **Drought Recovery (DR):** measurements from day 24 on included watered control, unwatered drought stressed and rewatered drought recovery plants.

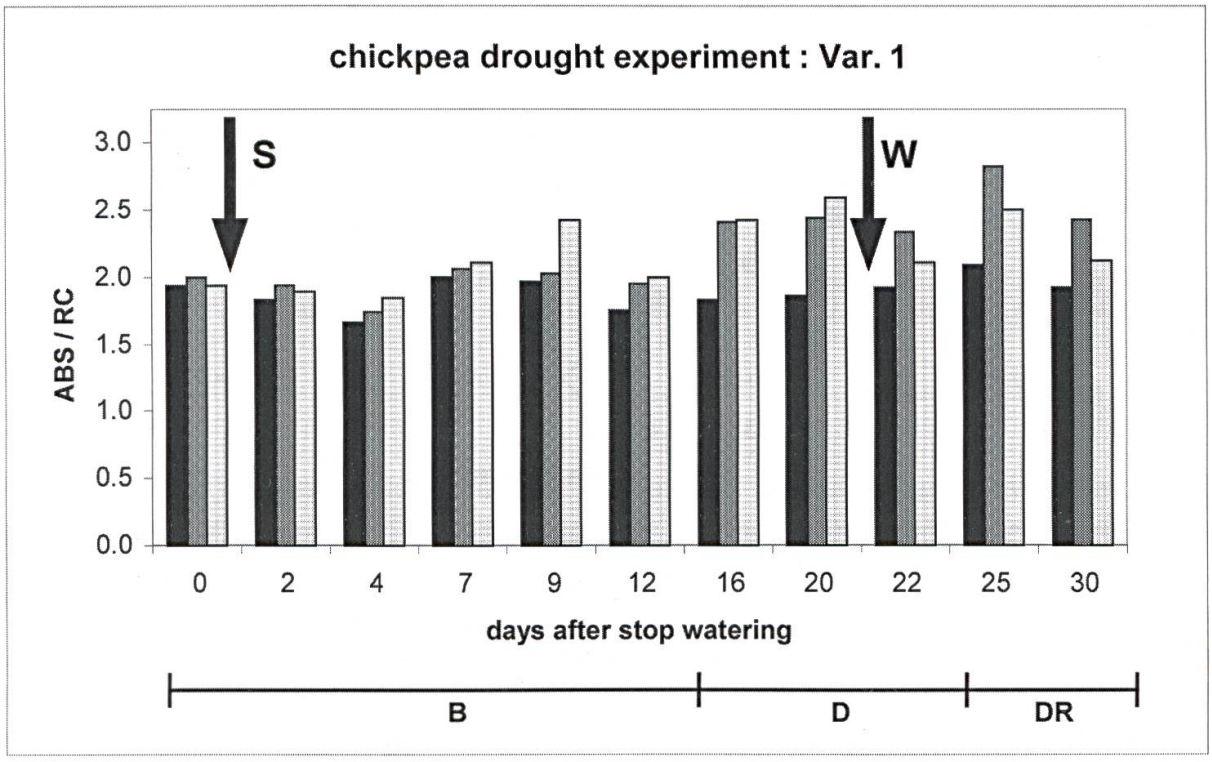

FIG. 2.
Absorption per Reaction Center of chickpea variety 1 (ICCV-10) along the drought experiment The left arrow S (day 0) indicate when watering was stopped for drought pots, the right arrow W (day 22) indicates start of rewatering the recovery pots. The phases B, D and DR indicate which data were pooled for data evaluation (see text).

control drought stress stress recovery

The quantum yield for PSII (φ_{Po}) is only slightly affected by drought stress for all 9 varieties (Fig. 3 top curves). It generally slightly decreased with drought and with most varieties increased again during recovery and almost reached the values of the control

plants. φ_{Po} of the varieties 1, 8 and 9 appear rather insensitive to drought. On the other hand the yield for electron flow ($\varphi_{Eo} = \varphi_{Po} * \Psi_0$) is highly responsive towards drought stress on all varieties (Fig. 3, bottom curves). This indicates that the probability of electron transport beyond Q_A^- (Ψ_0) is decreasing under drought stress (between 22 and 63%). Since the effects of drought stress conditions have increased the absorption per reaction center, while the absorption per cross section appeared reduced with time. This indicates inactivation of a part of the reaction centers.

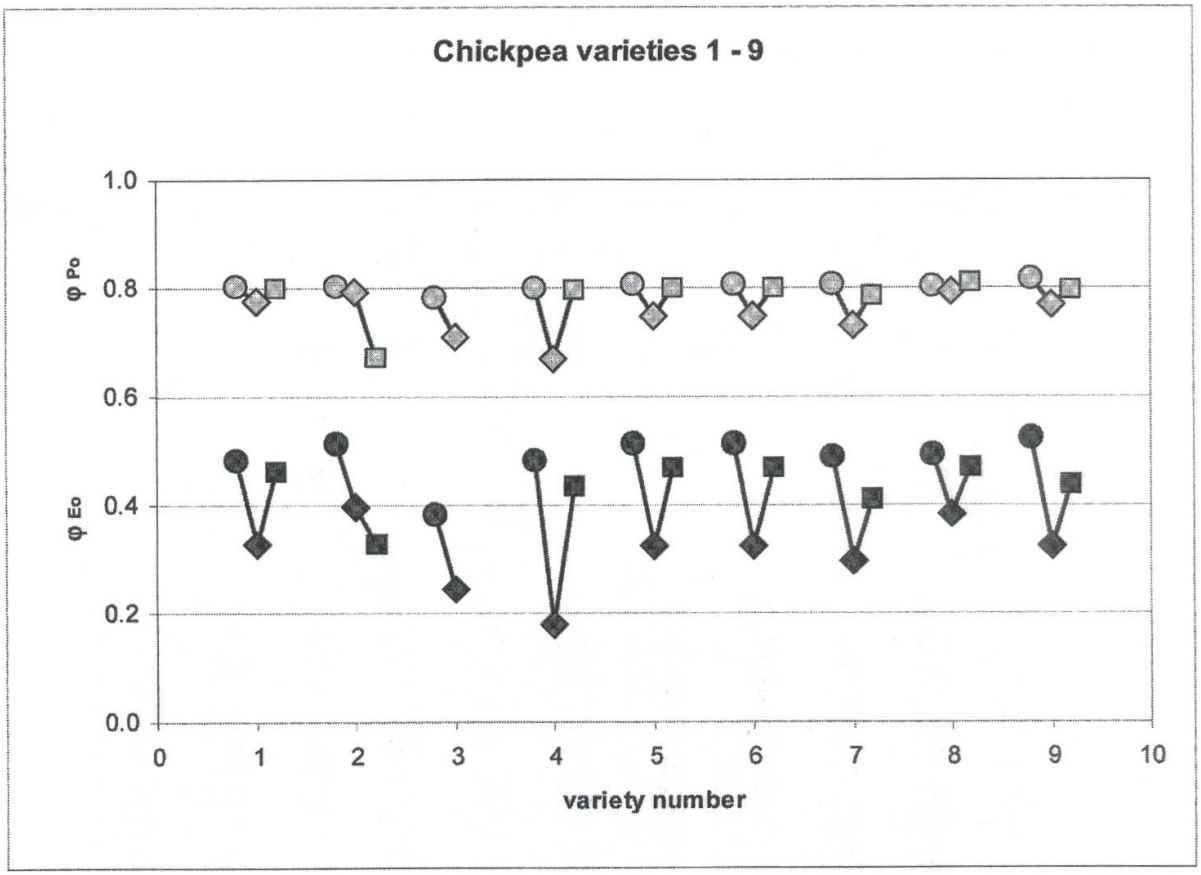

FIG. 3.
Effect of drought on quantum yield for primary photochemistry (φ_{Po}) and electron flux (φ_{Eo}) of photosystem II (○ control, ◇ stressed and □ recovering) during the recovery phase of 9 chickpea cultivars.

Symbols: ○ φ_{Po} ● φ_{Eo}

The effects of drought on the over all photosynthetic activity of plants have been analyzed by comparing the performance indices (PI) of the drought stressed plants with the control samples. As the described effects on Ψ_0 and RC/ABS have a cumulative effect on the performance index, significant changes have been observed in PI of all nine varieties. This is confirmed by strong changes in PI for all 9 varieties during DR phase, where the continued drought causes a severe drop in PI for all varieties (Fig. 4). In the same Figure it can also be seen that most varieties recovered during the rewatering period. Only var. 2 and 3 did not show any recovery: var. 2 exhibiting even lower PI than

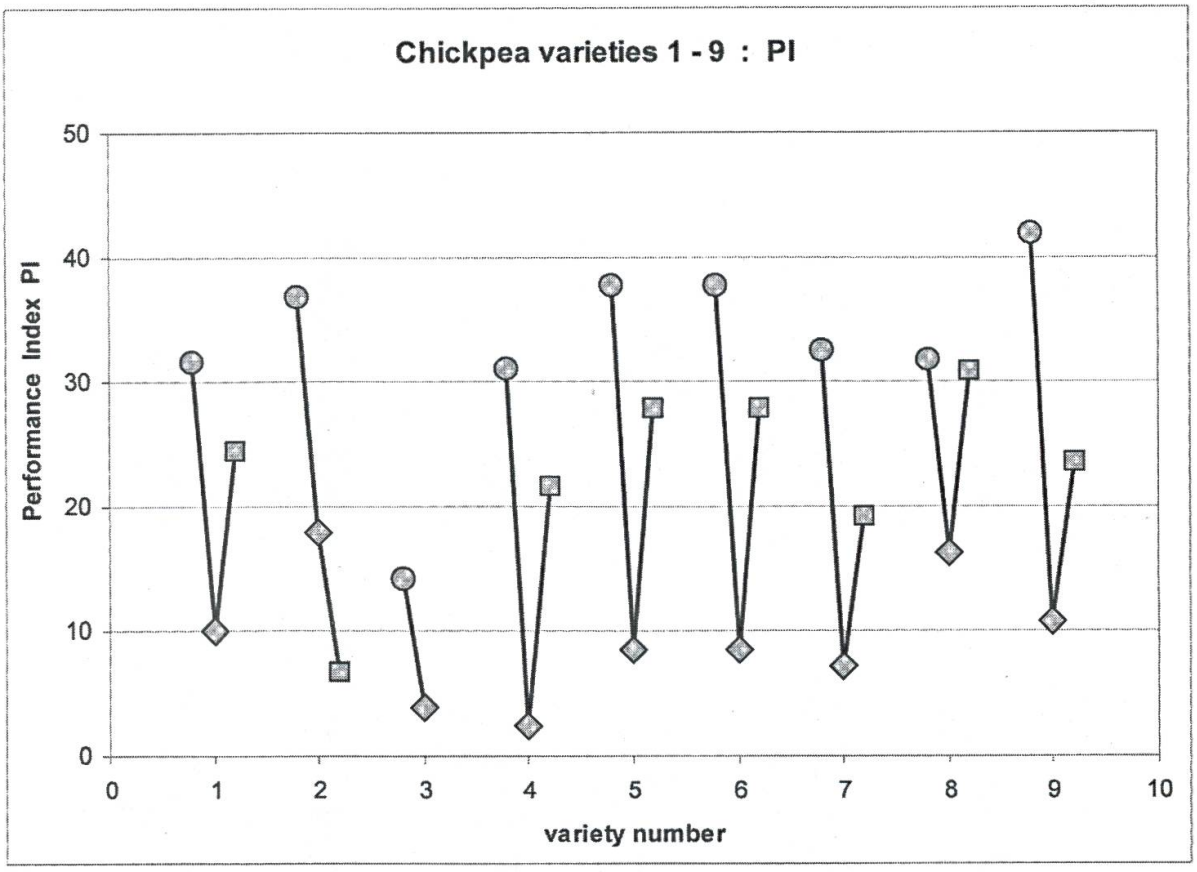

FIG. 4.

Effect of drought and recovery on the performance Index PI of 9 chickpea cultivars phase.

Symbols: ○ control ◇ stressed □ recovering

the continuously stressed plants, and var. 3 is anyway almost dying, as even the control plants show very low PI. On the other hand var. 8 almost recovered to the control level.

(b) Salinity Effects

From Fig. 5 in can be seen that stress symptoms on the PSII fluorescence behavior are much earlier recognized as for drought stress. Significant changes can bee observed seven to ten days after starting the NaCI treatment. Increased values for F_0 and a reduced ones for F_M were observed. Fig. 5 shows var. 5 (PANT G-114) as an example; the other varieties behaved similarly. The decrease of the F_M values was much more pronounced than the increase of F_0 values. Similarly to the drought experiment also for salt stress the measurements were pooled in 3 periods:

- **Before salt stress (B):** measurements from day 0 to 7 (all controls)
- **Salt stress (S):** measurements of day 10 and 15 included watered controls and salt stressed plants.
- **Salt stress Recovery (SR):** measurement from day 17 only included watered control, salt stressed and rewatered salt recovery plants.

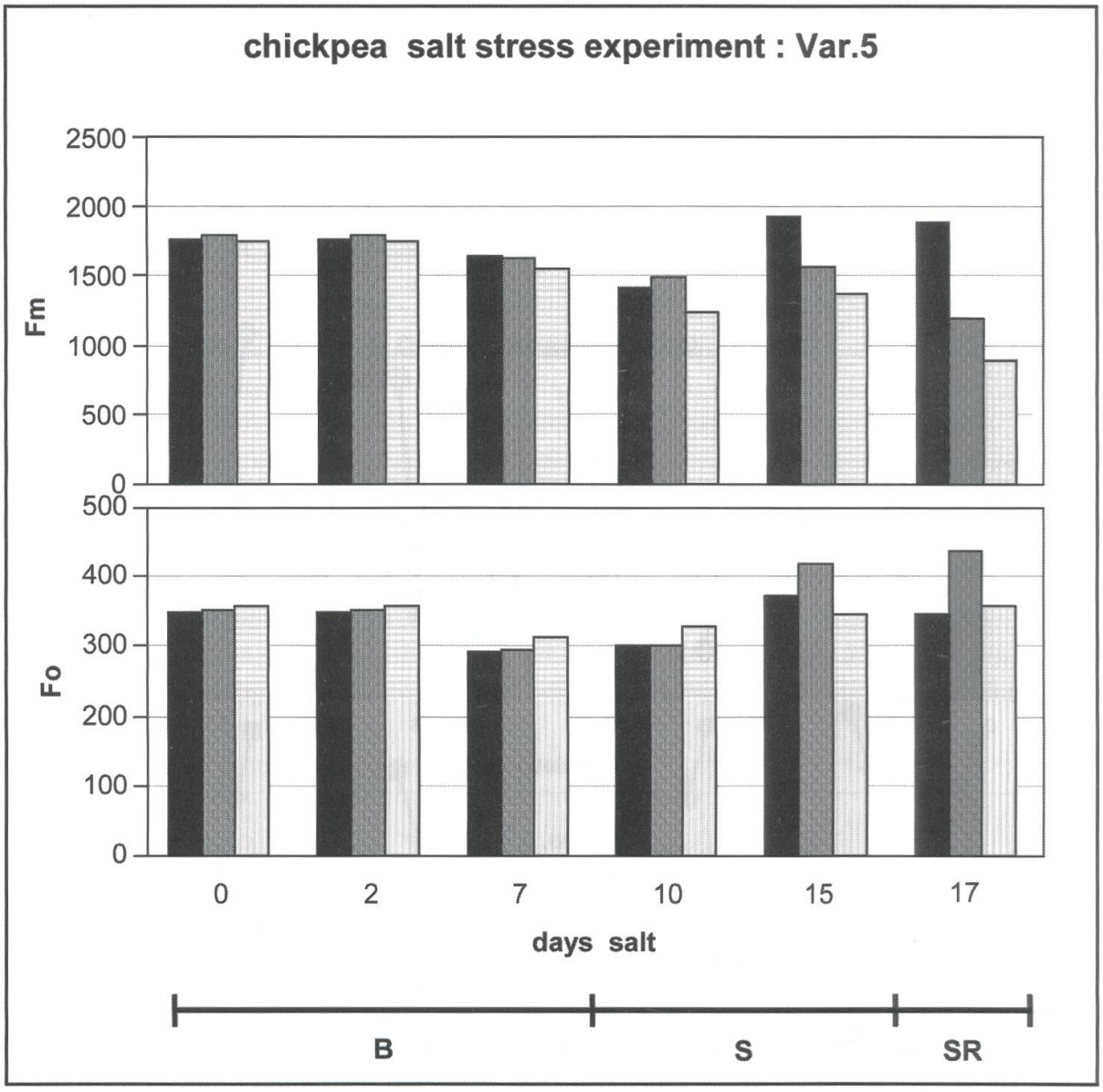

FIG. 5.

Fm (top) and Fo (bottom) of chickpea variety 5 (PANT G-114) along the salt stress experiment. Pooring NaCl (250 mM) started day 0 after measurement, rewatering for detecting recovery started day 14. The phases B, S and SR indicate which data were pooled for data evaluation.

control salt stress stress recovery

The high salinity condition effects to the oxidation of water that cause to change PSII photochemical efficiency and both Q_A^- and Q_B^- charge recombination with S states (MISRA *et al.*, 1999). This is reflected by the observation, that from day 10 after starting the salt treatments, quantum efficiencies and specific fluxes strongly differ from the non-treated control samples (Fig. 6).

Even though it has been reported that the quantum yield of the primary photochemistry of PSII is not affected by salinity stress (BRUGNOLI & LAUTERI, 1991; BRUGNOLI & BJORKMAN, 1992), but that it can be superimposed by high temperature stress

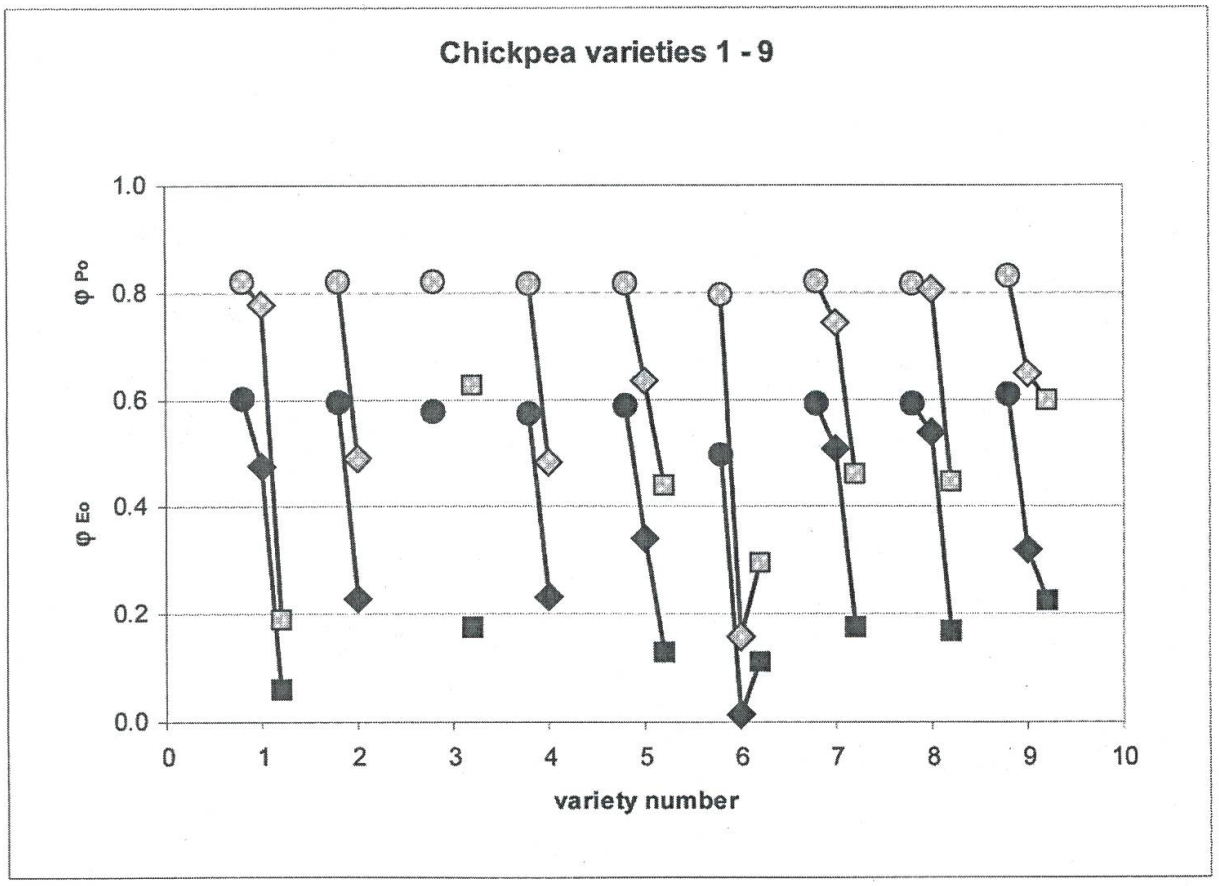

FIG. 6.

Effect of salt stress on quantum yield for primary photochemistry (φ_{Po}) and electron flux (φ_{Eo}) of photosystem II (○ control, ◇ stressed and □ recovering) during the recovery phase of 9 chickpea cultivars.

Symbols: ○ φ_{Po} ● φ_{Eo}

(LARCHER *et al.*, 1990) our results are in contradiction to that finding. The present study shows more variation in the φ_{Po} of salt stressed plants than the drought stress plants where φ_{Po} remained similar to the non-treated samples. However the electron transport per trapping (Ψ_0) and hence electron flow yield (φ_{Eo}) of drought stressed and salt stressed plants decreased strongly. Since the damages done by the salinity effects are comparably high, the average PI of the stressed and recovering plants appear much more reduced compared with the respective controls than those seen in the drought stress plants (Fig. 7). Under the conditions of this test none of the nine varieties tested has gained an ability to overcome the salt stress effects. It appears that pouring high amounts of NaCl solution to the soil additionally to the salt effect also gives rise to a depletion of oxygen in the root system (anoxia), to which chickpeas are reported to be severely sensitive (SINGH & SAXENA, 1999, pp. 66f).

Maturing and aging of the plants also causes changes in the photosynthetic mechanism (SRIVASTAVA & GOVINDJEE, 1999). It has to be expected that photosynthetic parameters as determined with the PSII fluorescence analysis also vary with age and development of the plants. As chickpeas are reported to be more sensitive to drought during pod

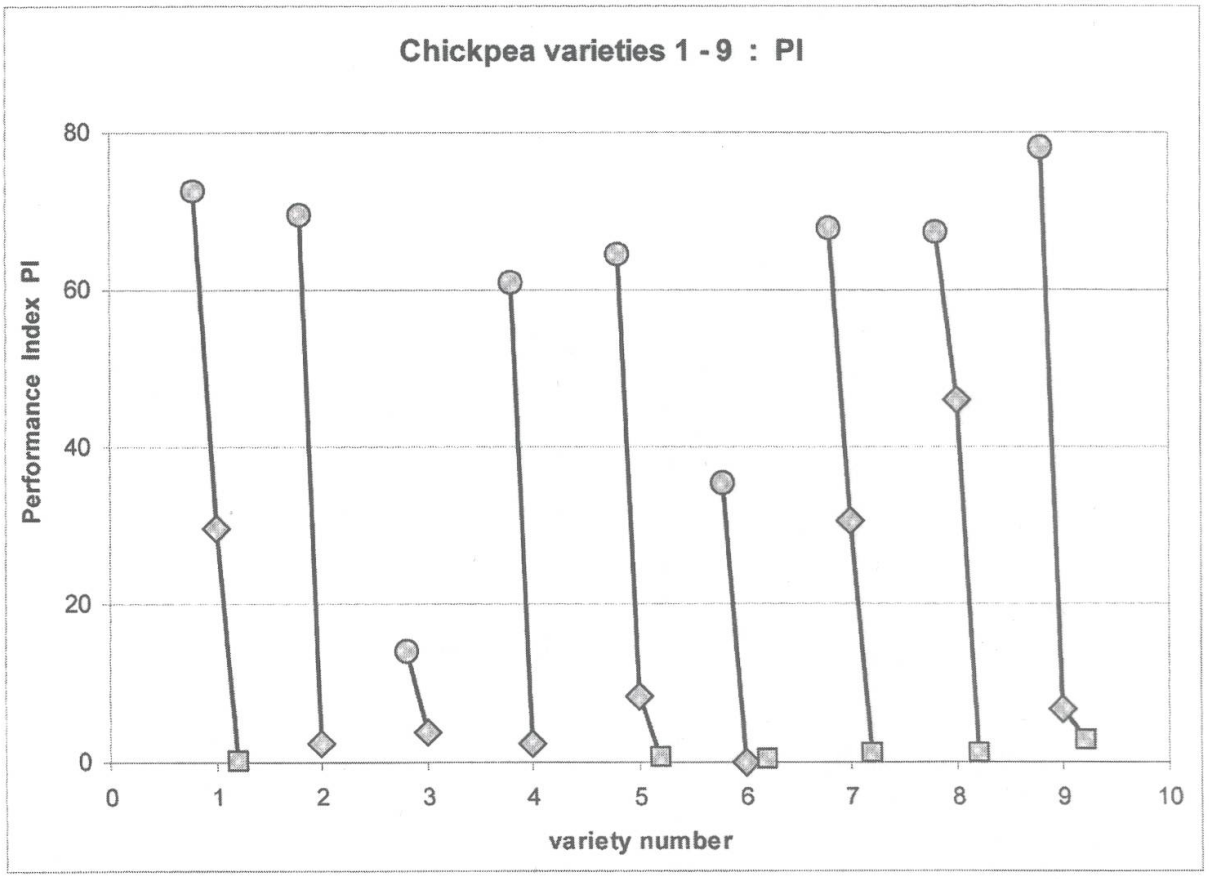

FIG. 7.
Effect of salt stress and recovery on the performance Index PI of 9 chickpea cultivars.
Symbols: ○ control ◇ stressed □ recovering

filling (SINGH & SAXENA, 1999, pp. 66f), one can suggest that also stress tolerance does change during development. Some of these developmental changes can be seen from Figs 2 and 5 where the parameters can be followed during the whole course of the experiment. There it becomes obvious that the values determined for the control plants change with time. As stated above the fluorescence analysis can indicate stress before visible symptoms apear. This preliminary observation however needs further investigation.

As shown in Fig. 8a all the varieties could withstand stress conditions for some time, and among them variety 1, 4, 5, 6, and 7 showed a quite high ability to tolerate both drought and salinity stress. However no significant recovery has been observed from any of the varieties that have undergone salt stress (Fig. 8b), whereas varieties 5, 6 and 8 exhibited high recovery potentials (75 to 100%), followed by varieties 1, 4 and 9 showing 50 to 70% recovery from drought stress.

From the results discussed above it can be concluded that the JIP-test can be a useful tool to detect differential stress tolerance potentials of different varieties. However it can also be concluded from these results that for chickpeas the method for testing salt stress tolerance has to be improved.

This study is part of ISCB program, project PS4.

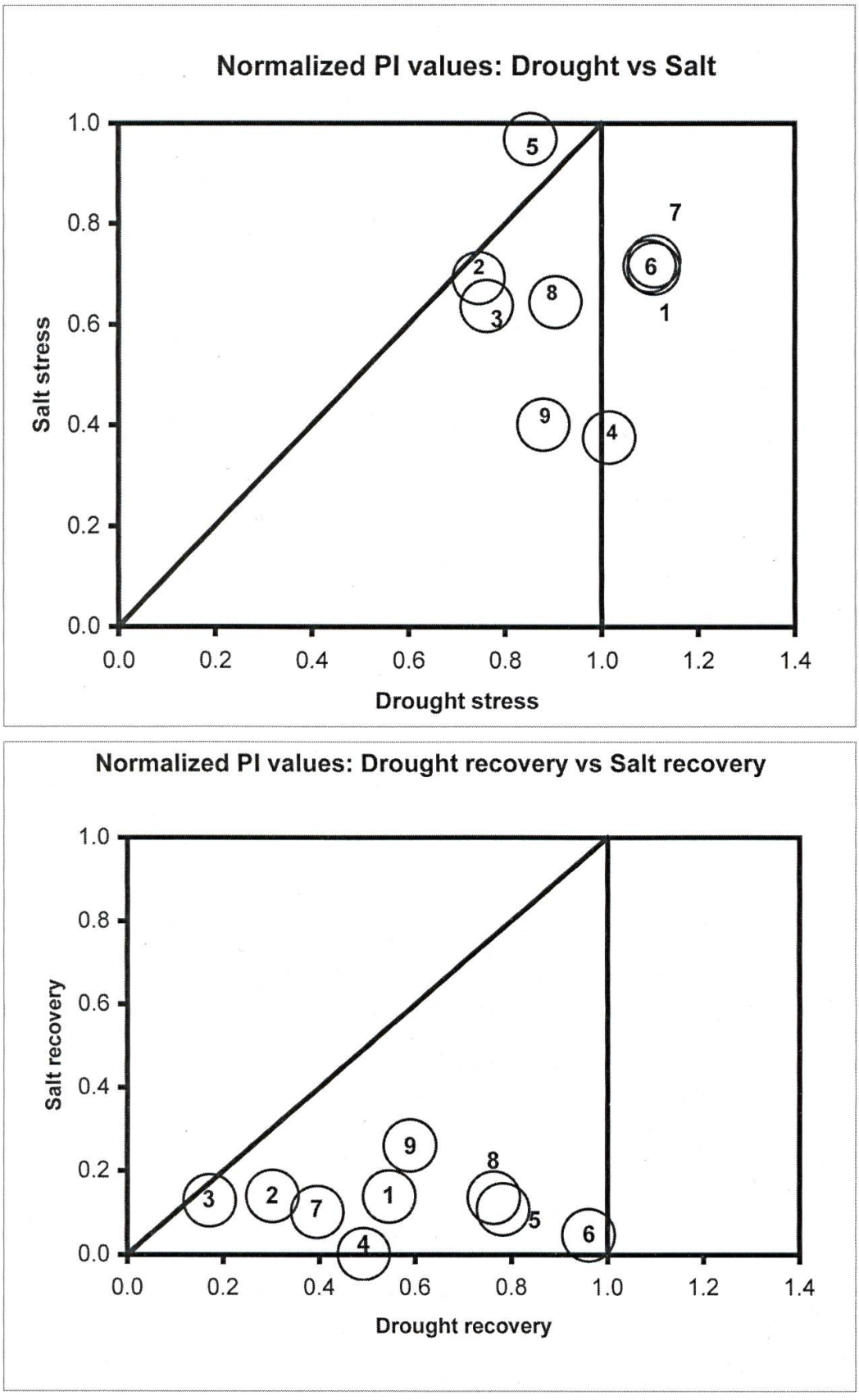

FIG. 8.

The normalized PI data of drought and salinity experiments were represented as a graph to select the most stress tolerable chickpea varieties among all nine varieties.

RÉSUMÉ

UTILISATION DE LA TECHNIQUE DE FLUORESCENCE RAPIDE POUR LA DÉTECTION DE VARIÉTÉS DE POIS CHICHE (*CICER ARIETINUM* L.) TOLÉRANTES À LA SÉCHERESSE ET LA SALINITÉ

L'analyse de la fluorescence chlorophyllienne est reconnue comme une méthode facile et rapide pour détecter les effets de différents stress (sécheresse, chaleur, froid, polluants atmosphériques, du sol ou de l'eau) chez les végétaux. Le présent travail évalue la possibilité d'utiliser cette technique pour comparer la tolérance de différentes variétés de pois chiche (*Cicer arietinum*) envers les stress de sécheresse et salinité. Les résultats confirment que, parmi les 9 variétés testées, il y a des différences majeures concernant la tolérance de stress pendant un certain temps. Quelques variétés ont aussi montré la capacité de se rétablir d'un stress de sécheresse; par contre aucun rétablissement du stress de salinité n'a été constaté.

La méthode choisie s'est montrée utile pour la comparaison de la tolérance différentielle de stress dans les plantes.

Mots-clés: Fluorescence chlorophyllienne, stress, sécheresse, salinité, JIP-test, index de performance photosynthétique.

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