Indian J. Plant Physiol., Vol. 10, No. 3, (N.S.) pp. 218-224 (July-Sept., 2005)

INDUCED WATER STRESS INFLUENCING PROLINE ACCUMULATION, PROTEIN PROFILES AND DNA POLYMORPHISM IN CHICKPEA CULTIVARS

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Received on 29 May, 2004, Revised on 19 Aug., 2005

SUMMARY

Five drought susceptible and five drought tolerant chickpea cultivars were evaluated for proline accumulation in addition to separation of stress responsive proteins by PAGE in relation to imposed water stress. The PCR based RAPD technique was employed to detect polymorphism in genomic DNA with eight random primers in each of the two drought susceptible and tolerant cultivars. Higher magnitude of proline accumulation was observed in the leaves of stressed plants of tolerant cultivars except in PG-5. In tolerant cultivars, an additional protein band of ~17.78 kD size was observed under water stress condition alongwith other protein bands of ~16.21, 36.30, 46.77 and 85.11 kD. The primers OPD-15 and OPC-09 detected polymorphism in genomic DNA of two tolerant and two susceptible cultivars.

Key words **: *Cicer arietinum*, PAGE, PCR, proline, random primers, RAPD, water stress**

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third important pulse crop in the world only after dry bean and dry peas. Among the chickpea growing countries, India ranks first in both area and production (Singh *et al.* 2002). However, the productivity in India is low as compared to other countries (Joshi *et al.* 2001, Kumar *et al.* 1997). In Maharashtra due to erratic rainfall, the productivity of chickpea is severely affected by drought besides several biotic constrains (Sharma and Ortiz 2000). Although drought management has been an option to obtain the realizable yields, it is increasingly felt that the genetic improvement of drought tolerance is more rewarding (Udaykumar *et al.* 1998). The seed based technology seems to be easier to transfer to farmers than the more complex knowledge based agronomic practices. Therefore, pulse breeders wish to develop drought tolerant cultivars of chickpea either by traditional breeding methods or by biotechnological approaches in order to minimize the losses occurring due to drought. It is also important to know the basic biochemical events *viz.*, perturbation of whole metabolic pathway leading to accumulation or depletion of metabolites, alteration in activities of conveniently assayable enzymes and changes in the pattern of synthesis of proteins of known function associated with drought tolerance (Hanson and Hitz 1982). The present study is mainly focussed on proline accumulation and protein profiles in relation to induced water stress in chickpea cultivars. Further, the lack of uniform drought stress in the field will render screening for drought tolerance ineffective and will limit progress from selection. Therefore, germplasm screening for tolerance to drought under naturally occurring drought stress does not seem to be reliable (Ortiz *et al.* 2002). Hence, the present investigation is an attempt to detect DNA polymorphism between drought susceptible and tolerant cultivars.

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MATERIALS AND METHODS

Seeds of five drought susceptible chickpea cultivars *viz*., PG-92926, PG-97110, Vishal, PG-12, PG-9414-7 and five drought tolerant chickpea cultivars *viz*., PG-96006, PG-9222-2, Vijay, PG-5, Chafa, which were obtained from the Principal Scientist, All India Co-ordinated Pulses Improvement Project, M.P.K.V., Rahuri. Ten seeds of each cultivar were sown in separate earthen pots in duplicate, containing about 10 kg soil and were maintained under glass house condition. One set of plants of each cultivar was regularly irrigated and served as a control. In another set, the plants of each cultivar were subjected to water stress by withholding irrigation after 30 days of sowing. The leaf samples from control and water stressed plants were collected at 45 days after sowing when water stress symptoms appeared. The soil moisture content from each pot was measured as per the method of Gardner (1986). The leaf portions of the water stressed and control plants were separated, cleaned and immediately subjected to biochemical analysis. Proline content in leaves was determined using acid ninhydrin reagent as per the method described by Bates *et al.* (1973) and expressed on fresh weight basis as μmoles g^{-1} fr.wt.

Soluble proteins from the leaves were extracted in 0.1M Tris-HCl buffer (pH 7.6) containing 5% β-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue (Laemmli 1970) and quantified by Lowry *et al.* (1951) method. Electrophoresis was carried out on 12% native PAGE (Davis 1964) and 12% SDS-PAGE (Laemmli 1970, Walker 1986). The band intensity was assessed visually by placing gel over a transilluminator for presence or absence of specific bands and recorded as faint, faint dark, dark and intense. The relative mobility (Rm) of resolved stress responsive proteins and protein molecular weight marker bands were measured. The molecular weights of stress responsive bands were calculated from the standard curve of protein molecular weight marker versus its Rm value.

For RAPD analysis, two susceptible *viz*., PG-92926, PG-97110, and two tolerant *viz*., PG-96006 and Vijay, chickpea cultivars were selected. Genomic DNA of these cultivars was isolated from the frozen fresh leaf tissues of 7-day-old seedlings using the miniprep method of Doyle and Doyle (1987). Purification and quantification of DNA was also carried out as described in Sambrook and Russell (2000). Eight random decamer primers (Operon Technologies, Inc. U.S.A.), were used to amplify genomic DNA. The RAPD analysis of the genomic DNA of four chickpea genotypes was carried out as per the protocol described by Naghia *et al.* (2002). PCR reactions were conducted in a volume of 25 μl containing 2.5 μl 10 x Taq buffer, 2.0 μl of dNTPs (0.5 each), 1.5 μl of primer (0.2 M), 0.5 μl of *Taq* polymerase (0.5 units), 0.4 μl $MgCl_2$, 2 μl of genomic DNA (25 ng) and 16.1 μl of sterile distilled water. The PCR reaction was performed in a thermal cycler (FGENO2TO, Techne Ltd., Duxford, Cambridge, U.K.). Amplified DNA fragments were electrophoresed on a 1.2% (w/v) agarose gel and visualized under UV-transilluminator and the gel was photographed on a gel documentation system.

RESULTS AND DISCUSSION

The results obtained in this investigation indicate differences in leaf proline content in susceptible and tolerant chickpea cultivars under unstressed condition (Table 1). After imposing the stress by withholding water, it was observed that the proline content was increased in each cultivar irrespective its drought tolerant or susceptible characteristics. The results revealed higher magnitude of proline accumulation and fold-increase in water stressed plants of tolerant cultivars with the exception of PG-5. The tolerant cultivars showed about 9.0 μmoles g^{-1} fr. wt. proline content under water stressed condition and ~12-fold increase over control. However, susceptible cultivars had proline content of ~7.6 μmoles g^{-1} fr. wt. under water stressed condition and ~9-fold increase over control. Based on the proline content under water stressed condition and fold increase over control, the cultivar PG-96006 appeared to be more drought tolerant than the rest of the drought tolerant cultivars. However, the cultivar PG-5 showed drought susceptibility based on the amount of proline accumulation (~7 μmoles g^{-1} fr. wt.). Several investigators have reported positive relationship between free proline content in leaves with drought tolerance in chickpea (Gupta *et al.* 1997, Singh and Singh 1999). Similar correlation was observed in the present investigation with the exception of cultivar PG-5. The higher magnitude of proline accumulation, may

Table 1. Effect of water stress on proline content (µmol g^{-1} fw) in leaves of chickpea cultivars

Cultivars	Control	Stress
Susceptible cultivars		
PG-92926	0.73 [38.30]	7.69 [21.09]
PG-97110	0.95 [37.40]	7.04 [20.12]
Vishal	0.99 [37.35]	7.63 [19.70]
PG-12	0.72 [38.10]	8.06 [19.70]
PG-9414-7	1.01 [38.10]	7.47 [20.60]
Tolerant cultivars		
PG-96006	0.73 [37.15]	9.41 [19.80]
PG-9222-2	0.78 [37.51]	8.90 [20.10]
Vijay	0.70 [38.72]	8.91 [19.60]
PG-5	0.93 [37.40]	6.88 [21.04]
Chafa	0.72 [37.20]	9.13 [20.40]
S.E. ±	0.013	0.035
C.D. at 5%	0.037	0.105

* The figures in parentheses indicate the % soil moisture content (% SMC).

help plants to tolerate dehydration by maintaining cell turgidity as reported earlier by Sivakumar *et al.* (1998) and may protect plants against singlet oxygen and free radical induced damages due to its action as a singlet oxygen quencher and scavanger of hydroxyl radicals. Proline is also able to stabilize proteins, DNA and membranes as evidenced earlier by Matysik *et al.* (2002). From the results obtained in the present investigation, it can be concluded that the chickpea cultivars which accumulated proline to the extent of 9.0 mmol g^{-1} fr.wt. or more may be classified as tolerant ones. Earlier researchers (Bates *et al.* 1973 and Sivakumar *et al.* 1998) have suggested that proline content under water stress can be used as an evaluating parameter for selecting drought tolerant varieties in crop plants.

The stress responsive proteins were resolved from the leaf portions of five susceptible and five tolerant cultivars of chickpea by native-PAGE (Fig. 1 and 2). A newly synthesized protein band of ~17.78 kD size was

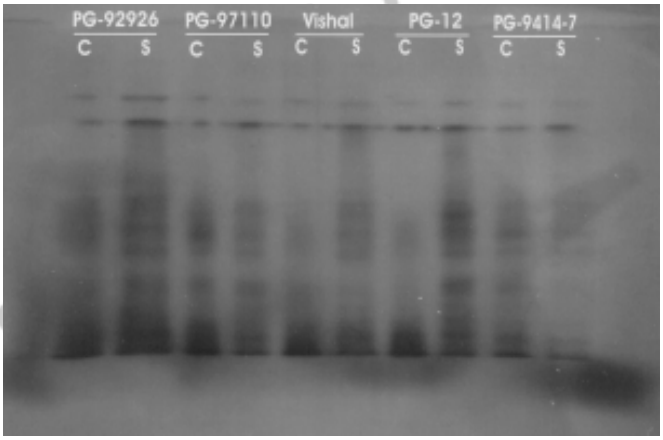

Fig. 1. Soluble proteins profile as resolved on 12% Native-PAGE from leaves of drought susceptible chickpea cultivars under control and stressed conditions. C=Control; S=Stressed

observed in tolerant cultivars under stressed condition except in PG-5 (Fig. 2). However, quantitative changes were observed in respect of bands of the size of ~22.38 kD, ~27.54 kD and ~58.88 kD size when resolved on

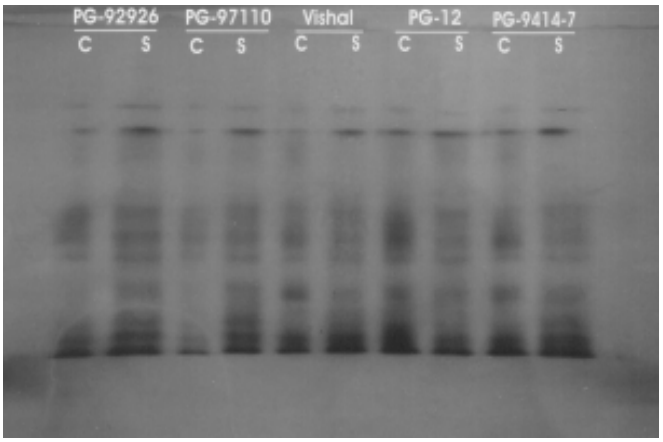

Fig. 2. Soluble proteins profile as resolved on 12% Native-PAGE from leaves of drought tolerant chickpea cultivars under control and stressed conditions. C=Control; S=Stressed

native-PAGE. When proteins were resolved on SDS-PAGE (Figs. 3 and 4), the bands of ~16.21kD, ~36.30

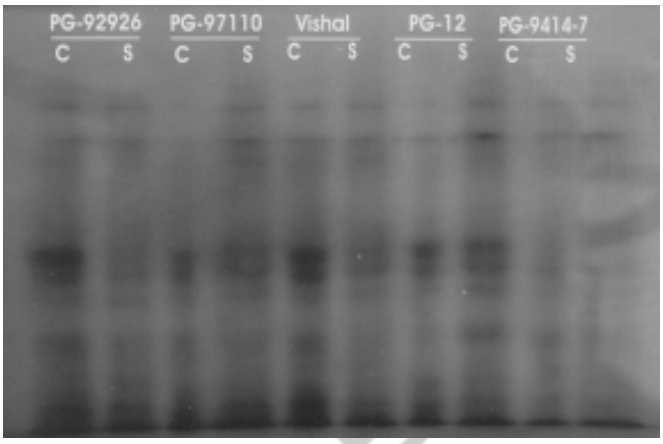

Fig. 3. Soluble proteins profile as resolved on 12% SDS-PAGE from leaves of drought susceptible chickpea cultivars under control and stressed conditions. C=Control; S=Stressed

kD, ~46.77kD and ~85.11 kD size appeared as newly synthesized ones in tolerant cultivars (Fig. 4). Tyagi *et al.* (1995) observed a polypeptide of 22 kD size under

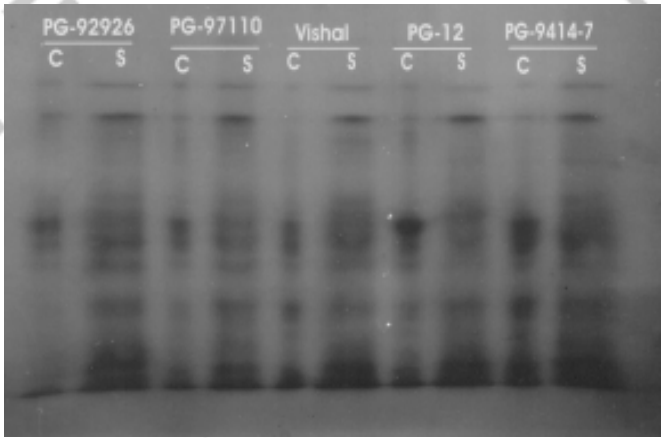

Fig. 4. Soluble proteins profile as resolved on 12% SDS-PAGE from leaves of drought tolerant chickpea cultivars under control and stressed conditions. C=Control; S=Stressed

stressed condition in *Lathyrus sativus*. In the present investigation, a protein band of ~22.38 kD on native-PAGE appeared only in tolerant cultivars under water stressed condition (Fig. 2). Osmotin gene encoding 26 kD protein has also been expressed during the salt stress and water stress in tobacco (Singh *et al.* 1987). In the present investigation, a ~27.54 kD protein was resolved on native-PAGE and appeared as a dark band and its intensity increased during stressed condition in tolerant cultivars of chickpea indicating over expression of the gene encoding it (Fig.2). Close *et al.* (1993) identified four related barley dehydrin proteins of 22.6 kD, 16.2 kD, 14.4 kD and 14.2 kD and 17 kD size in maize. In the present investigation, a protein of ~16.21 kD size appeared as a newly synthesized when resolved on SDS-PAGE (Fig. 4) and a band of ~17.78 kD appeared when resolved on native-PAGE (Fig. 2). These bands were observed in tolerant cultivars of chickpea. Lee *et al.* (2002) reported 16 and 18 kD bands as major protein bands induced by drought stress in white clover. Thus, the results obtained in the present investigation are in agreement with the results reported by earlier researchers. A previous study by two-dimensional electrophoresis of proteins synthesized under water stressed conditions revealed no major qualitative differences as compared to unstressed (control) in maize seedling mesocotyls. However, several quantitative differences were evident, with some proteins exhibiting more intense synthesis under stress and some with reduced synthesis (Bewley *et al.* 1983). The results of this investigation are in agreement with the earlier report of Bewley *et al.* (1983). Shinozaki and Yamaguchi-Shinozaki (1997) reported that genes induced during drought stress are thought to function not only in protecting the cells from water deficit by the production of important metabolic proteins but also in the regulation of genes for signal tranduction in drought stress response.

The RAPD analyses of the genomic DNAs of two drought tolerant and two drought susceptible cultivars of chickpea were performed by employing a total of eight random primers to detect polymorphism between these cultivars. Only five primers *viz.* OPD-08, OPC-05, OPC-06, OPC-07 and OPC-11, amplified monomorphic bands. However, two primers *viz.*, OPD-15 and OPC-09 generated the polymorphic bands (Fig. 5-7). The primer OPD-15 synthesized an unique fragment of the size of 2.84 kb in two drought tolerant chickpea cultivars, PG-96006 and Vijay, while remaining absent in susceptible cultivars, PG-92926 and PG-97110 (Fig. 5, lanes 5-8). The primer OPC-09 depicted the polymorphism, however, the bands of 1.35 kb and 1.27 kb size were generated in one of the tolerant (PG-96006) and one of the susceptible

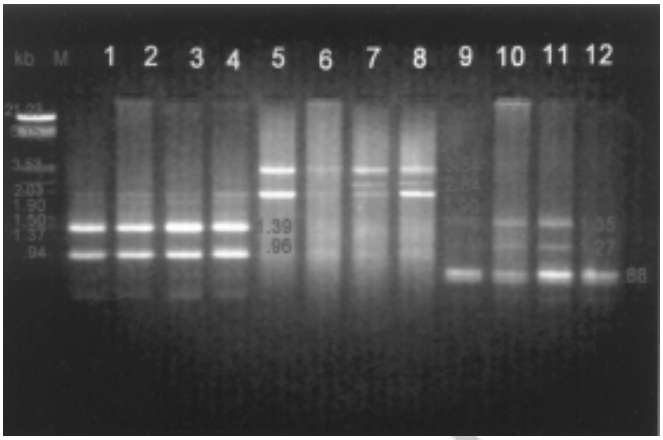

Fig. 5. RAPD analysis of chickpea genomic DNAs by random primers. Random primers OPD-08 (lanes 1-4), OPD-15 (lanes 5-8) and OPE-09 (lanes (9-12). Lanes 1,5 and 9-PG-92926; lanes 2,6 and 10 - PG-97110; lanes 3,7 and 11 - PG-96006; lanes 4,8 and 12 - Vijay; M-DNA molecular weight marker, *Eco* RI + *Hind* III double digest.

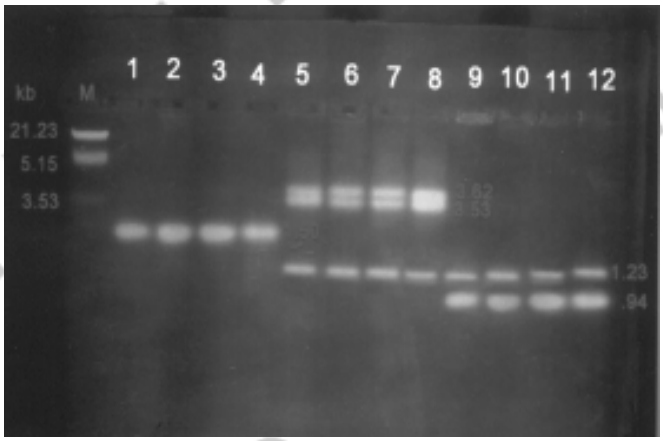

Fig. 6. RAPD analysis of chickpea genomic DNAs by random primers. Random primers OPC-05 (lanes 1-4), OPC-06 (lanes 5-8) and OPC-07 (lanes 9-12). Lanes 1,5 and 9 - PG-92926; lanes 2,6 and 10 - PG-97110; lanes 3,7 and 11 - PG-96006; lanes 4,8 and 12 - Vijay; M-DNA molecular weight marker, *Eco* RI + *Hind* III double digest.

(PG-97110) cultivars while remaining absent in other susceptible and tolerant cultivars (Fig. 5, lanes 9-12). The primer OPC-09 synthesized the polymorphic bands of 4.23 and 3.52 kb size in two tolerant cultivars, PG-96006 and Vijay, while remaining absent in the susceptible cultivar, PG-97110 (Fig. 7, lanes 1-4). Thus, it appears

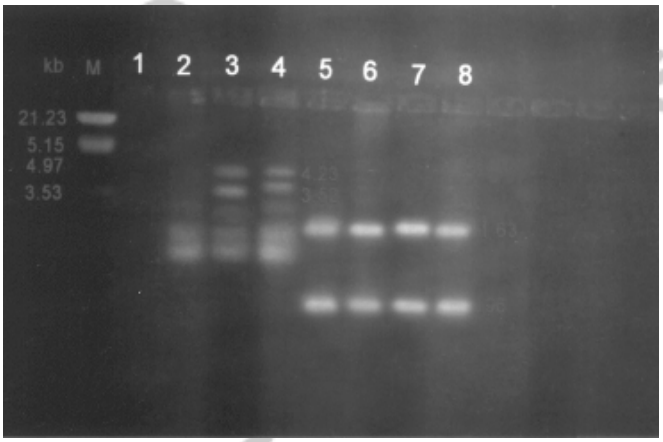

Fig. 7. RAPD analysis of chickpea genomic DNAs by random primers. Random primers OPC-09 (lanes 1-4) and OPC-11 (lanes 5-8). Lanes 1 and 5 - PG-92926; lanes 2 and 6 - PG-97110; lanes 3 and 7 - PG-96006; lanes 4 and 8 - Vijay; M-DNA molecular weight marker, *Eco* RI + *Hind* III double digest.

that primers OPC-09 and OPD-15 may be used for screening the tolerant and susceptible cultivars of chickpea. Earlier, Singh *et al.* (2002) detected polymorphism with forty of the hundred random primers employed by them in 23 chickpea genotypes. Out of a total of eight random primers used in the present investigation, only two detected polymorphism. Singh *et al.* (2002) revealed that most of the primers produced single polymorphic bands with only 14.12% of polymorphism, however, in the present investigation, the polymorphism to the extent of 20-50% was observed.

This study concludes that large increase in proline accumulation in tolerant chickpea cultivars may be considered as important adaptive characteristics under water stress. RAPD analysis of genomic DNA revealed that the primers, OPC-09 and OPD-15, synthesized unique fragments of the size of 4.23, 3.52 and 2.84 kb only in tolerant cultivars. The protein bands synthesized in response to water stress are thought to be stress responsive and are immensely important for further biotechnological research.

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