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Effect of Drought Stress on the Concentration of Nitrogen Metabolites in White Clover

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ABSTRACT: To investigate the changes in the composition and pool size of nitrogen metabolites under drought stress, white clover (*Trifolium repens* L.) were exposed to -0.04MPa (well-watered, control) or to -0.12MPa (drought-stressed) of soil water potential during 28 days. Dry weight of leaves in drought-stressed plants was remarkably decreased by 45% and 74% within 14 days and 28 days, respectively, compared with control. For nitrate concentration after 28 days of treatment, a significant difference (1.6 times higher in drought-stressed plants) was observed only in stolon. NH_3-NH_4^+ concentration in all three organs of drought-stressed plants linearly increased to more than 1.6 times higher level at 28 day when compared to the initial level (day 0), while the increasing rate in control was much less than that of drought-stressed plants. Proline concentrations in drought-stressed plants remarkably increased and reached to 7, 13 and 17 times higher level at 28 day compared to control. Protein concentration in leaves of drought-stressed plants tended to decrease, while it slightly increased during the first 14 days and reached a plateau afterward in control. There was not significant difference in the proteins concentration of stolon and roots throughout experimental period. On SDS-PAGE analysis, two major proteins specifically induced by drought stress (16-kD and 18-kD) were detected in stolon.

Keywords **:** Drought stress, White clover, Nitrate, NH_3-NH_4^+, Proline, Proteins

White clover (*Trifolium repens*, L.) is the most important forage legume in the temperate climate zone (Søegaard, 1994). It has been known that white clover has a low tolerance to drought because of its shallow root system. A prolonged drought, predominantly in spring of Korea, can kill a stand and high temperature throughout the following summer is also unfavorable for growth. The consequence of poor sward clover content leads often to the problems in agricultural practice. However, the mechanisms underlying the inhibition of plant growth by drought are not yet fully understood.

It is well documented that nutrient uptake from soil is inhibited in dry soil. Soil drought decreased mineralization of organically bound nutrients (Bloehm *et al.*, 1992; Walworth, 1992), and nutrient transport in soil by mass flow and diffusion (Seiffert *et al.*, 1995), and thus may diminish nutrient availability at the root surface. Severe drought may further decrease nutrient transport to the root surface by inducing root shrinkage and consequently results in loss of soil-root contact (North and Nobel, 1977). During prolonged periods of drought, the decrease in water availability for transport-associated processes leads to changes in the concentrations of many metabolites, followed by disturbances in amino acid and carbohydrate metabolism. For example, there is an increase in the synthesis of compatible solutes such as special amino acids (Lazcano-Ferrat and Lovatt, 1999; Rabe, 1999), sugars (Bohnert *et al.*, 1995; Lee *et al.*, 2002; Kim, 2000), and Gly betaine (Yancey *et al.*, 1982; Girousse *et al.*, 1996). Acclimation to drought requires responses that allow essential reactions of primary metabolism to continue and enable the plant to tolerate water deficits.

Based on data in the literature, Rabe (1999) postulated that any stress condition causing reduced growth or impaired plant health will result in ammonia accumulation in the stress period and suggested that the detoxification process in which excess ammonium in the cells is removed results in the accumulation of nitrogen-containing compounds, such as putrescine and proline. The elevated ammonia levels during water stress (Hake and Lovatt, 1987) and low temperature (Zheng and Lovatt, 1987) in *Citrus* species are additional evidence of altered N metabolism during stress.

It is widely documented that proline level increases in response to drought. A 3- to 300-fold increase has been described in various species and treatments (Delauney and Verma, 1993). Proline accumulation has been proposed as a mechanism for storage of excess N (Stewart and Boggess, 1977). Proposed roles in stress tolerance of proline include serving as osmotica, stabilizing macromolecules and membranes, and detoxifying tissues of excess N (Huang *et al.*,

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1994). Despite of extensive research to elucidate the roles of proline in ameliorating the detrimental effects of abiotic stresses on plant growth and yield, the physiological significance of their accumulation under water deficit remains equivocal.

The aims of this study were 1) to determine the changes in the pool size of nitrogen metabolites in response to drought stress 2) to find out a useful indicator of plant health and the intensity of stress injury in relation to stress-inhibited plant growth. In addition, SDS-PAGE was carried out to detect the drought-induced proteins.

MATERIALS AND METHODS

Plant culture and experiment procedure

Sods of white clover (*Triforium repens* L.) at full vegetative stage were transplanted to 3 L pot containing a mixture of sand and fritted clay. The soil in all pots was watered to field capacity during 2 weeks of adaptation. Soil water potential (Ψ_s) was determined by a tensiometer (Jet Fill, Soil Moisture Equipment Corp., Santa Barbara, U.S.A). The range of Ψ_s was chosen to expose plants to moderate to severe level of drought stress, showing leaf wilting at midday. Control pots were watered every day to maintain constant soil water potential (Ψ_s) close to 0.04 MPa. Drought stress was imposed by withholding water until Ψ_s of -0.12 MPa, was achieved. Each treatment lasted for 28 days. Plant sample were harvested at 14 and 28 days after treatment, respectively. Harvested plants were separated into leaves, stolon and roots. Tissue samples were immediately frozen in liquid nitrogen. Freeze-dried samples were finely ground and stored under vacuum for further analysis.

Chemical analysis

Total N was determined by ammonia microdiffusion on Conway dish as described by Kim and Kim (1996). An aliquot of 0.5 mL of digest and 4 mL of distilled water were pipetted into test tube, and then 0.5 mL of Nessler's ammonium color reagent was subsequently added. After mixing thoroughly and 10 min of coloration, the absorbance at 410 nm was read. N amount was calculated by the correlation equation between absorbance and N-NH_4^+ content

Nitrate was extracted with 80% (v/v) ethanol. About 200 mg of finely ground freeze-dried sample was homogenized with 25 mL of 80% ethanol and 1 mL of 0.1N H_2SO_4 was added to avoid ammonium volatilization. The combined aqueous samples were placed on the horizon shaker and agitated at 250 rpm for 1hr. The extracts were filtered through a whatman No.2 filter paper. Nitrate in aqueous extract was determined as described by Cataldo (1975)

For determination of NH_3-NH_4^+ concentration, about 200 mg of sample was immediately homogenized with 10 ml of 10% (w/v) trichloroacetic acid and then centrifuged at 10000 *g* for 10 min at 4°C (Lazcano-ferrat and Lovatt, 1999). After microdiffusion of the acid soluble supernatant fraction, containing the combined pool of NH_3-NH_4^+ as NH_4^+, in Conway dishes, colorimetric determination of NH_4^+ was performed with Nessler's reagent on an aliquot of solution.

Proline was extracted and its concentration was determined by the method of Bates *et al.* (1973). Leaf segments were homogenized with 3% sulfosalicylic acid and the homogenate was centrifuged at 3000 *g* for 20 min. The supernatant was treated with acetic acid and acid ninhydrin, boiled for 1 h and then absorbance at 520 nm was determined. Proline concentration was calculated using L-proline as a standard.

Proteins were extracted by suspending freeze-dried sample with 100 mM sodium phosphate buffer (pH 7.0). Tubes were vortexed for 30 sec 4 times and centrifuged at 13000 *g* for 10 min, and the supernatant was retained. The pellet was re-extracted. Soluble proteins in the combined supernatant were quantified using dye-binding method (Bradford, 1976).

For SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) analysis, 20 proteins were separated in 1.5 mm thick gels containing 12.5% acrylamide (propenamide) (Laemmli, 1970) and stained with Coomassie Brilliant Blue R-250.

RESULTS

Dry weight

Leaf growth of control plants continued throughout exper-

Table 1. Changes of dry weight (g $plant^{-1}$) in leaves, stolon and roots of well-watered or drought-stressed plants during 28 days. Each value is the mean ± S.E. for n=3.

Organs	Days after drought treatment		
	Day 0	Day 14	Day 28
Leaves			
Well-watered	16.64 ± 0.47	20.94 ± 0.42	31.39 ± 3.26
Drought-stressed	16.23 ± 0.52	11.44 ± 0.03	8.31 ± 0.76
Stolon			
Well-watered	9.54 ± 0.08	11.97 ± 1.26	11.82 ± 1.17
Drought-stressed	9.62 ± 0.13	11.63 ± 1.43	10.73 ± 1.26
Roots			
Well-watered	5.65 ± 0.10	6.35 ± 0.22	8.97 ± 0.91
Drought-stressed	5.45 ± 0.31	6.25 ± 0.53	11.03 ± 1.06

imental period, showing about 2-fold increase in dry weight (Table 1). Withholding water for 28 days reduced leaf DW approximately 48% compared to the initial level (day 0). Between days 14 and 28, leaf DW accumulation was severely inhibited in drought-stressed plants : decrease by 45% within 14 days and 74% after 28 days of drying compared to well-watered plants. Dry matter of stolon was not affected by water treatment throughout experimental period. Dry matter of root in drought stress plants was not significantly different compared to control within 14 days, thereafter a stimulated increase of drought-stressed roots appeared.

Total N

Changes in total N concentration in leaves, stolon and roots of drought stressed or well-watered (control) plants were shown in Fig. 1. Total N concentration in leaves of drought-stressed plants was not significantly varied during 28 days, while it increased by 33.5% within 14 days in control. In stolon, total nitrogen concentration was less varied in both treatments. Total N concentration in roots of drought-stressed plants was not significantly changed, whereas in control plants total N continuously increased during 28 days. Withholding water for 28 days reduced root N about 31% when compared to control.

Nitrate

Nitrate concentrations in drought-stressed and control plants are given in Fig. 2. Nitrate concentration in leaves of both treatments slightly increased, and showed non-significant difference at any sampling dates. Nitrate concentration in stolon of control plants continuously decreased to 67% of the initial level, while slightly increased (15%) within 14

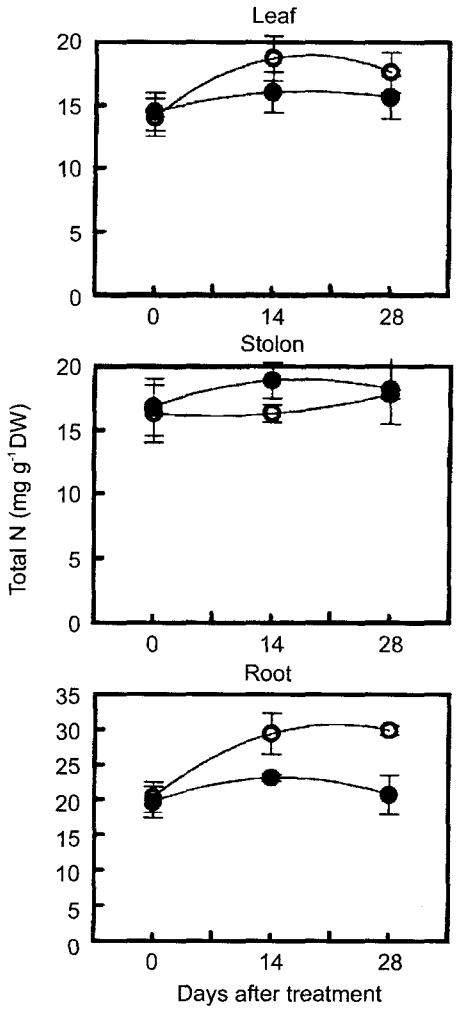

Fig. 1. Changes on total nitrogen concentration in leaves, stolon and roots of well-watered (○) or drought-stressed (●) plants during 28 days. Each value is the mean ± S.E. for n=3.

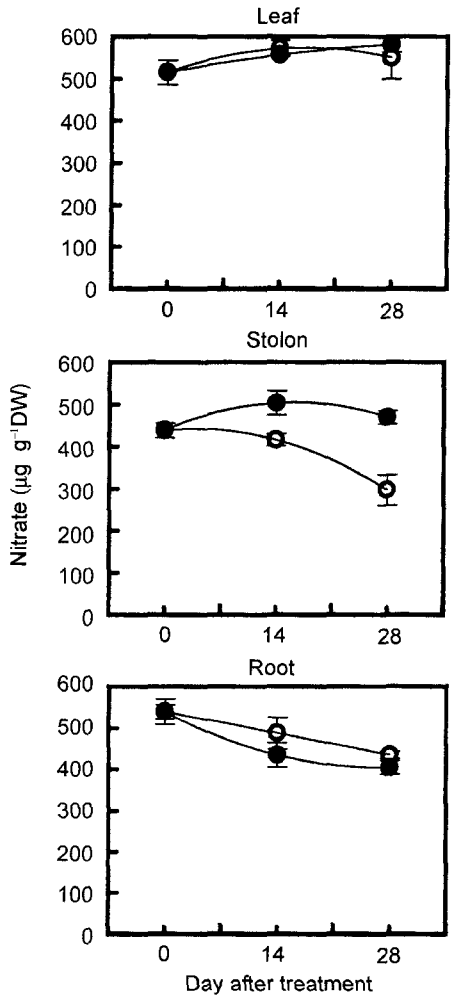

Fig. 2. Changes on nitrate concentration in leaves, stolon and roots of well-watered (○) or drought-stressed (●) plants during 28 days. Each value is the mean ± S.E. for n=3.

days in drought-stressed plants. In roots of both treatments, nitrate concentration decreased during 28 days and reached at 80% of the initial level.

NH_3-NH_4^+

Changes in NH_3-NH_4^- concentration in leaves, stolon and roots of drought stressed or well-watered (control) plants were shown in Fig. 3. Leaf NH_3-NH_4^+ concentration in drought-stressed plants linearly increased and reached to 2-times higher level at 28 day after drying when compared to the initial level (day 0). However, it significantly increased only for the first 14 days (about 28 % increase) and then leveled off in control plants. In stolon, the concentration was remarkably increased by 14 days of drought treatment and then slightly decreased. NH_3-NH_4^+ concentration in stolon of control plants also tended to increase, but the increasing rate was much less compared to drought-stressed plants. NH_3-NH_4^+ concentration in roots of drought-stressed plants linearly increased, whereas it was relatively stable in control. NH_3-NH_4^+ concentration at day 28 in drought-stressed roots was 1.5 times higher than that of control.

Proline

Proline accumulation was found to be one of distinct symptoms induced by drought stress, showing remarkable increases in all three organs examined (Fig. 4). The proline concentration of leaves in drought-stressed plants was largely increased by 3.5 times within 14 days and 7.8 times within 28 days compared to initial level. In contrast, leaf proline concentration in control plants was not significantly changed during 28 days. Similar trends were observed in stolon and roots of drought-stressed plants. Comparing the

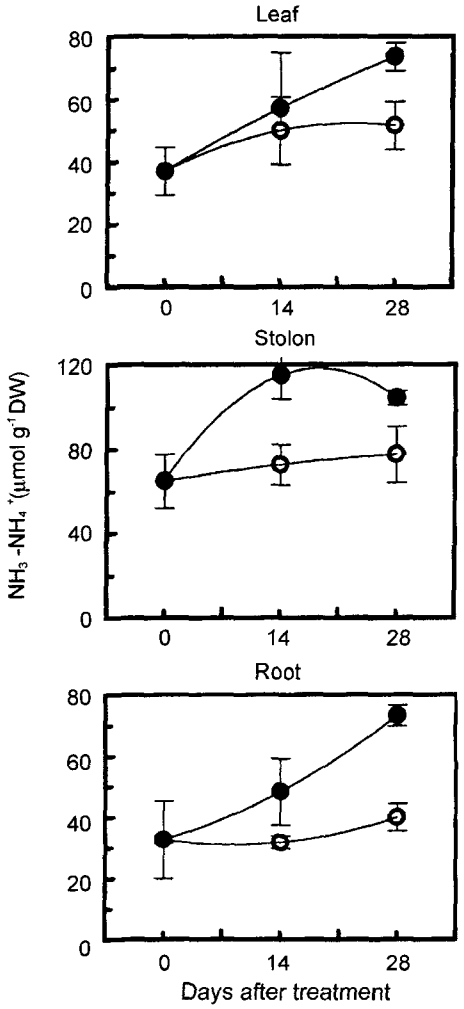

Fig. 3. Changes on NH_3-NH_4^+ concentration in leaves, stolon and roots of well-watered (○) or drought-stressed (●) plants during 28 days. Each value is the mean ± S.E. for n=3

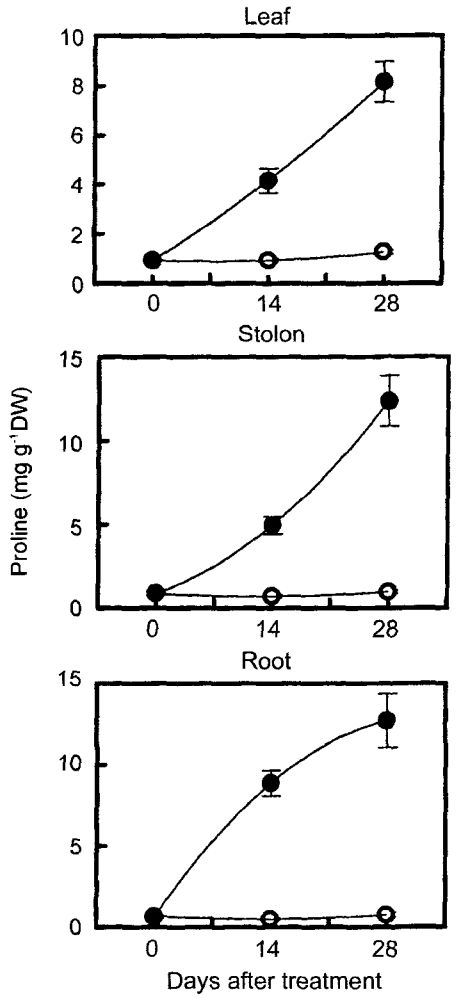

Fig. 4. Changes on proline concentration in leaves, stolon and roots of well-watered (○) or drought-stressed (●) plants during 28 days. Each value is the mean ± S.E. for n=3

values of control at 28 day after treatment, it was estimated that drought increased proline accumulation about 13 and 17 times, respectively, in stolon and roots.

Proteins

Changes in protein concentration in leaves, stolon and roots of control or drought-stressed plants during 28 days after treatment are shown in Fig. 5. Protein concentration was less varied in both drought-stressed and control plants. Concentration of leaf proteins in drought-stressed plants tended to decrease, while in control plants slightly increased during the first 14 days and reached a plateau afterward. In stolon, protein concentration in control slightly increased only for 14 days and then decreased, while it was not significantly changed in drought-stressed plants. There was not

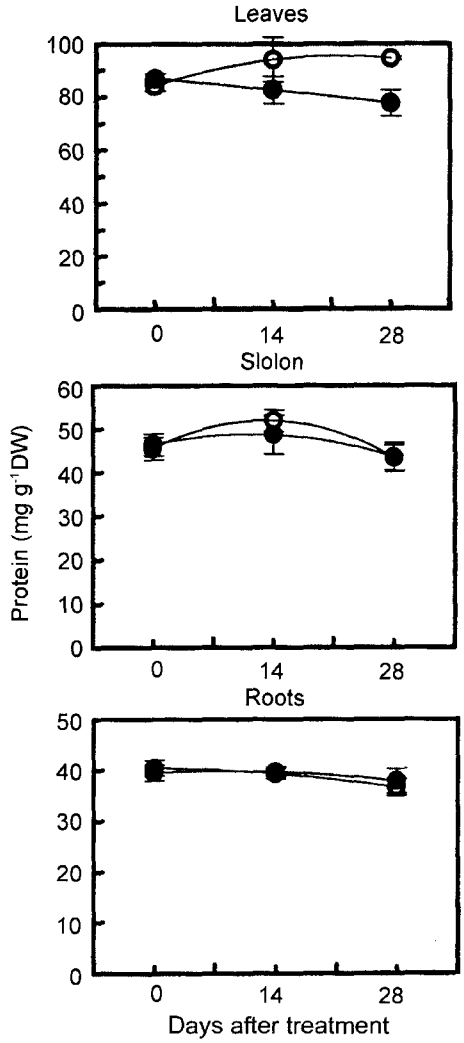

Fig. 5. Changes on protein concentration in leaves, stolon and roots of well-watered (○) or drought-stressed (●) plants during 28 days. Each value is the mean ± S.E. for n=3.

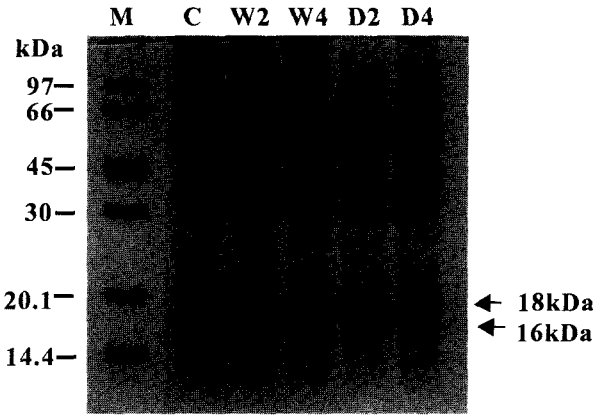

Fig. 6. Sodium dodecylsulfate polyacrylamide gel electrophoresis analysis of buffer-soluble proteins extracted from stolon of well-watered or drought-stressed plants. Sample of 20 g proteins was loaded in each well. C: 0-day control, W2 : harvested at 14 day from well-watered plants, W4 : harvested at 28 day from well-watered plants, D2 : harvested at 14 day from drought-stressed plants, D4 : harvested at 28 day from drought-stressed plants.

significant difference in root protein concentration between control and drought-stressed plants throughout experimental period.

The variation in the protein pattern of stolon tissues of control (W2 and W4) and drought-stressed plants (D2 and D4) was assessed by SDS-PAGE analysis (Fig. 6). In control plants, the protein pattern at 14 day (W2) and 28 day (W4) showed similar polypeptide composition with the initial pattern (C, day 0). In drought-stressed plants, two polypeptides with relative molecular masses of 16- and 18-kD started to accumulated at 14 day of drought (Fig. 6, D2) and remained at a higher level until 28 day (Fig. 6, D4). However, the drought-induced difference in polypeptide composition was not observed in leaf and root tissues (data not shown).

DISCUSSION

The most important agronomic problem for perennial forage species growing under prolonged drought is the decrease of production. This is confirmed in the present study. Leaf dry weight of drought-stressed plants decreased by 45% within 14 days, and 74% within 28 days compared with well-watered control plants (Table 1). It has been reported that in several cultivars of forage grasses leaf expansion rate was halved within 5 days of drought, and had almost ceased within 10 days (Volaire *et al.*, 1998).

Twenty-eight days of drying made a significant difference in nitrate concentration in stolon (Fig. 2), which is a main storage site of organic reserves (Collins *et al.*, 1991). In con-

trol plants, showing a continuous leaf growth, nitrate concentration remarkable decreased during 28 days. These results indicated that leaf growth inhibition induced by drought stress led to the decrease in N transport to sink organ (e.g. leaves in this case) and resulted in high nitrate level in storage organ (stolon). It has been documented that water stress causes decrease in NO_3^- absorption by the roots and transport from the root to the leaves (Shaner and Boyer, 1976; Larsson, 1992). Such decline in drought-stressed plants resulted in the accumulation of NH_3-NH_4^+ in three organs (Fig. 3) and the decrease in protein concentration in leaves (Fig. 5). These results indicate that the inhibition of protein synthesis in drought-stressed plant closely related to the accumulation of NH_3-NH_4^+ in plant tissues. The elevated ammonia levels have been found under water deficit (Lazcano-Ferrat and Lovatt, 1999; Yang and Kao, 2000), low temperature (Zheng and Lovatt, 1987) and phosphorus deficiency (Rabe and Lovatt, 1986). Given that inhibition of leaf growth of drought-stressed plants, further assimilation of NH_3-NH_4^+ to meet protein demand was much limited under drought stress. The metabolic basis for decreased protein concentration during water deficit is presently unknown. Whether lower concentration of proteins reduced leaf growth, or resulted from it, remains unsolved. In Recent, Rabe and Lovatt (1986) and Lazcano-Ferrat and Lovatt (1999) provided evidence that abiotic stresses that reduce leaf or shoot growth increase production of ammonia, leading to ammonia accumulation or its removal through biosynthesis of arginine, which is a precusor of proline (Stewart and Boggess, 1977).

Our results showed a continuous and sharp increase in proline concentration in all three organs of drought-stressed plants, but little changes in control plants (Fig. 4). This suggests that the degradation of proline was almost completely inhibited under water deficit condition. This suggestion is in agreement with the observation of Lazcano-Ferrat and Lovatt (1999) and Yang and Kao (2000) who showed that the decrease in proline utilization contributes to the accumulation of proline under water-stressed condition. As shown Fig. 4 and Fig. 5, the concentration of proteins did not correlated with proline accumulation among three organs examined of drought-stressed plants, suggesting that drought-induced proline accumulation was not dependent on inhibition of protein synthesis or increased protein degradation. Therefore, it could be suggested that the accumulation of proline in water-stressed plants is predominantly due to *de novo* synthesis. In addition, the inhibition of leaf growth (Table 1) accompanied with a sharp increase in proline concentration (Fig. 4) in drought-stressed plants, suggesting that proline accumulated under stress did not improve leaf growth. This leads to an assumption that proline accumulation is a symptom of stress. Proline accumulation in response to temperature extremes and salinity stress could be due to a disturbance in tissue water status comparable to that observed during drought stress (Aspinall and Paleg, 1981). A more common denominator among all these stress conditions may be that they result in a general reduction in growth rate when proline accumulates. Considering the fact that there is no feedback inhibition for N uptake and nitrate reduction even under stressed-condition (Miflin and Lea, 1980), the inhibition of protein synthesis in leaves (Fig. 5) and shoot growth (Table 1) in drought-stressed plants suggests that the lack of anabolic processes (protein synthesis and growth) leads to ammonia accumulation and subsequent detoxification by sequestering ammonia to nitrogen-containing compounds such as proline in this study.

Studies conducted by various investigators related to the effects of water stress on protein synthesis in many important crops suggest that water stress severely affected protein synthesis, alters gene expression and protein profiles in stressed- tissues, and induces the synthesis of stress induced-specific proteins (Dubey, 1999). On SDS-PAGE analysis, two major proteins specifically induced by drought stress (16-kD and 18-kD) were detected in stolon (Fig. 6).

In conclusion, these studies suggest that NH_3-NH_4^+ and proline might provide the useful indicators of plant health and the intensity of stress injury in relation to drought stress-inhibited plant growth.

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