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FREE PROLINE ACCUMULATION IN YOUNG SUGAR BEET PLANTS AND IN TISSUE CULTURE EXPLANTS UNDER WATER DEFICIENCY AS TOOLS FOR ASSESSMENT OF DROUGHT TOLERANCE

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ABSTRACT

Proline is the most widely distributed metabolite that accumulates under various stress conditions, including the lack of water. To evaluate the suitability of proline accumulation triggered by drought stress to screen sugar beet genotypes for drought tolerance, we analysed accumulation of free proline in eleven genotypes classed in three levels of relative tolerance (low, medium, high), as assessed visually in field cultivation. Analysis was performed in two tests: 1) in greenhouse, where 90 days old plants were exposed to a short-term water deficiency and 2) in tissue culture where the lack of water was imposed by addition of 3 or 5% (w/v) polyethylene glycol (PEG, MW 6000). Both the *in vitro* test with increasing levels of PEG and the suspension of water supply in the greenhouse experiment showed large increases of free proline in tissues of sugar beet explants or leaves consequent to water restriction, as well as reduction in fresh weight, tissue water content and axillary bud formation. Stress effects varied considerably among genotypes classed at low, medium and high levels of field tolerance to drought stress, but were similar as class averages, except for proline *in vitro*, which was significantly higher for genotypes in the high tolerance group, and allowed separating them from those in the less tolerant groups. Proline response in the *in vitro* test correlated better than the response in greenhouse experiment with the field assessed drought tolerance of genotypes.

Key words: free proline, sugar beet, tissue culture, PEG, drought.

INTRODUCTION

Plant responses to various environmental stresses are among the most attractive topics in plant science, because they directly influence plant growth and overall crop productivity (Qin et al, 2011; Luković et al., 2009). Plant adaptation to drought is a complex process, involving far more changes than just reduced growth (Conde et al., 2011). It implies, at the cellular level, specific regulation of gene expression, including genes encoding transport proteins (i.e. H+ pumps and Na+/H+ antiporters), increase in antioxidant activity, transient increase in ABA concentration, suppression of energy-consuming pathways and accumulation of compatible solutes and protective proteins (Bartels and Sunkar, 2005; Chaves et al., 2009). All these changes at the cellular level are critical to restore ion homeostasis caused by any given abiotic stress. The production of osmolytes, such as fructans, proline and glycine betaine, to modulate osmotic pressure, has been shown to be an effective means of enhancing plant abiotic stress tolerance in sugar beet. Those compounds are frequently accumulated as compatible solutes in plants and the expression of genes corresponding to some of the relevant enzymes also increase accordingly (Conde et al., 2011). Both cold and lack of water can cause problems in water uptake and hence provoke water stress in plant cells.

The ways by which plants adjust their metabolic level properly and differentially is being intensively investigated (Qin et al., 2011), but it is not still completely clear if the

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genotypes which accumulate these osmolytes under stress conditions better tolerate drought or not (Ghoulam et al., 2002; Yamada et al., 2005; Vallyiodan et al., 2006).

Although proline is the most widely distributed metabolite that accumulates under stress conditions (Delauney and Verma, 1993), the significance of this accumulation in osmotic adjustment in plants is still debated and varies from species to species (Hoai and Shim, 2003). The crystallization of sugar in the industrial processing of beet root in sugar refineries may be jeopardized by accumulation of compounds, such as proline and glucose, because they lead to the formation of coloured components that reduce the quality of beet roots (Campbell, 2002; Coca et al., 2004; Monreal et al., 2007).

There is experimental evidence that suggests that proline accumulation is a symptom of stress caused by injury, and not an indicator of tolerance to stress (Liu and Zhu, 1997). The correlation between the degree of stress and proline concentration suggests, indeed, that the accumulation of proline really is useful indicator of stress in sugar beet (Iannucci et al., 2000; Ain-Lhout et al., 2001; Putnik-Delic et al., 2010). Proline can act as a signalling molecule to modulate mitochondrial functions, influence cell proliferation and trigger specific gene expression, which can be essential for plant recovery from stress (Al-Khayri, 2002; Szabados and Savoure, 2009). There are a number of studies considering the impact of environmental stress on the growth of sugar beet, conducted under controlled conditions and in the field (Qi et al., 2005; Kenter et al., 2006). To the best of our knowledge, proline accumulation in plants grown under semi-controlled conditions and in plants grown by tissue culture method has not been compared so far. Water deficit as limiting factor in sugar beet production may cause important loss in sugar yield (sometimes more than 40%). To overcome this problem it is challenging to find selection criteria that are quickly and efficiently applicable in the breeding process. Therefore, the aim of this study was to analyse the production of proline in sugar beet under different experimental conditions and find out whether it can be used as relevant criterion for distinguishing between genotypes that differ in their capacity to tolerate water deficit.

MATERIAL AND METHODS

The study was conducted with eleven sugar beet (*Beta vulgaris* subsp. *vulgaris* L.) genotypes, numbered 1 to 11, that had been previously assessed in field trials for tolerance to water deficit. The assessment was based on observation test where the capability of genotypes to maintain turgor pressure after at least 7 days long period without precipitation was evaluated. Plants were observed during vegetation period when water shortage is usually critical for sugar beet production in Vojvodina (July and August). This observation permitted allocation of sugar beet genotypes into three groups (scale 0-5): low 0-2 (genotypes: 2, 5, 6 8), medium 3-4 (genotypes: 3, 7, 9, 11) and high 5 (genotypes: 1, 4, 10).

Greenhouse experiment

Sugar beet plants were grown in semi-controlled conditions of a greenhouse in the pots (31x37x13 cm). Ten plants per pots were grown in three replications. Substrate was a mixture of soil (2/3) and sand (1/3), with daily watering to maintain 80% field water capacity (FWC) and 16/8 h light/dark periods in a completely randomised design, with the eleven genotypes per two levels of the water stress treatment (no, yes). After 90 days, when plants had 8 to 10 fully formed leaves, water deficit was imposed by the cessation of watering, while the control plants continued to be watered up to 80% of FWC. Five days later the youngest completely formed leaves from ten plants per genotype were sampled for proline analysis. Root, stem and leaf dry weight (DW) was measured after drying samples to constant mass.

Tissue culture experiment

In a separate experiment the same sugar beet genotypes were grown *in vitro*. Seeds were surface sterilized and germinated on MS medium (Murashige and Skoog, 1962) to which were added 0.3 mg/l BA (benzyl

adenine) and 0.01 mg/l GA3 (gibberellic acid) (Mezei et al., 2006). Multiplied explants were transferred to the fresh medium every three weeks, until sufficient number of axillary shoots of comparable size was obtained. The buds were then placed on micropropagation media containing 0 (control), 3 or 5% (w/v) polyethylene glycol (PEG, MW 6000) (Duchefa, Netherlands), with experimental units of four explants in four replications. The cultures were maintained at 21-23°C, under 16 h illumination. After four weeks, the explants were used for analysis of buds fresh weight, dry weight, number of axilary buds and concentration of free proline.

Proline analysis

Proline concentration in leaves of plants from the greenhouse experiment and in axillary buds from tissue culture experiment was determined according to the method described by Bates (1973). Approximately 1g of plant material was homogenized in 10 ml 3% aqueus sulfosalicyclic acid and filtered through Whatman's filter paper. Two millilitres of filtrate were mixed with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1h at temperature of 100°C. The reaction mixture was extracted with 4 ml toluene, the chromophore containing toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520 nm with a Beckman, USA Duferies 60 spectrometer, using appropriate proline standards.

Statistical analyses

Mean responses to water stress per tolerance class and prediction response scores per genotypes were fitted with a mixed model including genotypes as a random factor and tolerance class x stress treatment (no/yes watering suspension or PEG concentration) as fixed factors.

Water stress effects *in vitro* and in soil on dry weight, percent water content and proline production for the sample of sugar beet genotypes were compared in terms of effect size (difference between the stressed and control response values divided by the standard deviation of the response variable). To this purpose the *in vitro* experiment data were also analysed retaining only the 0 and the 5% PEG concentration levels, as a factor corresponding to the stress factor of the greenhouse experiment. Natural logs of proline values were used to reduce the skewness of the distribution. Confidence intervals for fitted mean responses were calculated as quantiles of simulated distributions of the expected response values. Analyses were done with the R environment (R Development Core Team, 2012) and the contributed packages lme4 (Bates et al., 2011) and ggplot2 (Wickham, 2009)

RESULTS AND DISCUSSION

Greenhouse experiment

The water stress treatment applied to the greenhouse grown plants caused a noticeable, though not statistically significant, average decrease of plant fresh and dry weight, similar for the three levels of drought tolerance, but some genotypes in the low (2 and 5) and medium (3) levels were less affected than the others (Figure 1).

Water loss from plant tissues was significant only for the low and high tolerance levels, but genotype effects differed within each level, with the most severe losses shown by three genotypes (2, 5, 6) in the low and two (1, 4) in the high tolerance groups. As a result of water loss, the proportion of dry weight, of both root and above-ground parts, was increased by water stress for the low and high tolerance groups, except the genotypes 6 in the low group and 10 in the high group.

The intermediate group showed a very small average response in the same direction, due to contrasting response of genotype 11, which was the best in reducing water loss. Proline accumulation was stimulated by the stress treatment in all genotypes and particularly in some of the low (2, 6) and high (4) tolerance groups, with increases ranging between 160% and 90 times and average

increases higher for the low tolerance group (15 times) than for the medium and high ones (4 and 6 times, respectively). The least responsive genotypes were in the medium (9, 11) and high (10) tolerance groups.

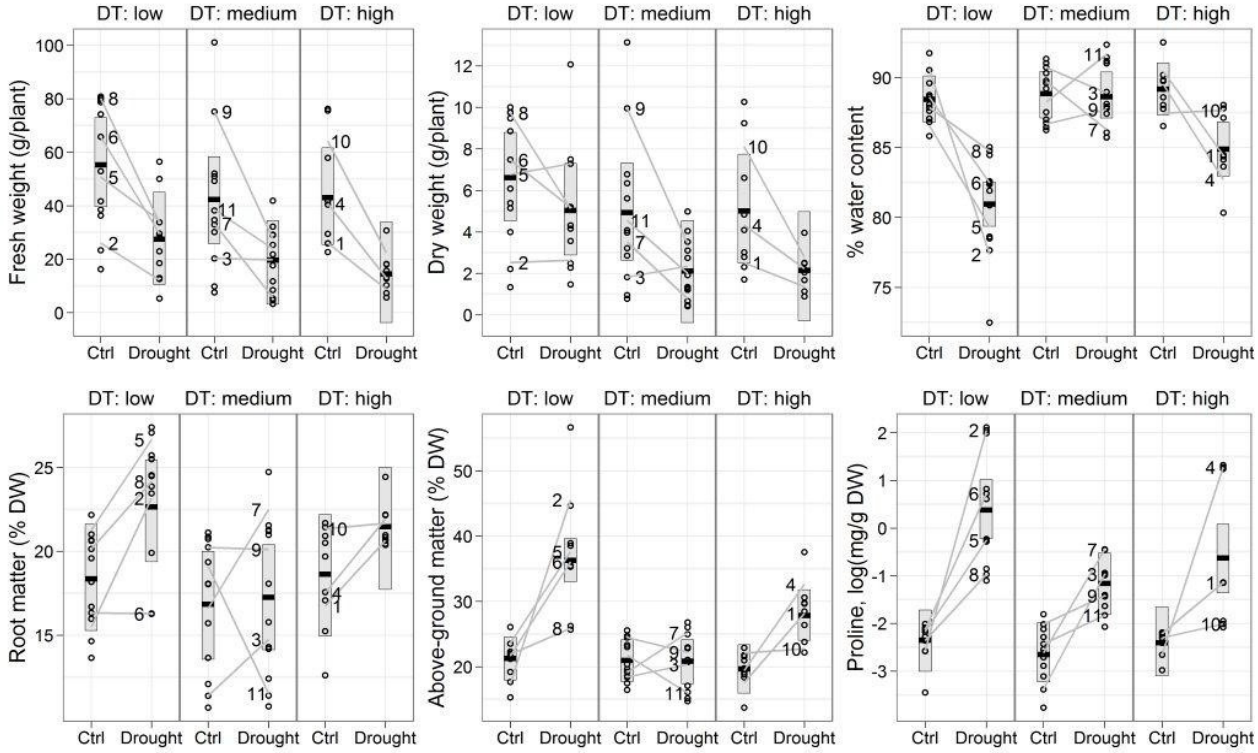

Figure 1. Effects of drought stress on growth traits and proline production of greenhouse grown plants of sugar beet genotypes (1 to 11) from three classes of visually field-assessed drought tolerance (DT). Observed values of three replicates (circles, ten plants each), average genotype positions (numbered grey lines) and class means with 95% confidence intervals (crossbars). The drought stress was induced by suspension of watering to test plots after three months of culture and observations were made after five more days.

The increase in proline accumulation under drought stress has shown positive correlation with growth at the cellular and seedling stages in wheat and rice (Shah et al., 2002; Song et al., 2005). In maize, proline content increased as the drought stress progressed and peaked after 10 days, and then decreased under severe water stress, as observed after 15 days (Anjum et al., 2011). In our greenhouse experiment the accumulation of proline appeared clearly associated to the condition of drought induced stress, but did not discriminate among genotype responses in a way correlated with visual assessment of drought tolerance on plants in the field. The disagreement may be partly due to the quality differences between the visual field observations, where moreover the water stress condition could be not so neat and temporally limited as the one induced in this experiment, and the instrumental measures used in the latter.

***In vitro* experiment**

The fresh weight of sugar beet explants cultured for one month in a growth medium containing up to 5% PEG decreased similarly with increasing PEG rate for the drought tolerance groups of genotypes, but the weight loss for the highest PEG rate was slightly higher, more than 50%, for the low tolerance group (Figure 2).

High tolerance genotypes showed parallel trends, but genotypes 2 in the low and 7 in the medium groups lost less weight than the others and lower development in absence of PEG. The average response of explants dry weight to PEG increase was not linear, showing a peak at 3% PEG and a drop to about the same levels observed in absence of PEG at 5% PEG. The curvature was a little more marked for the low tolerance group and genotype trends were roughly parallel, except for the linear trend of genotype 9, even if varying widely in level of dry weight.

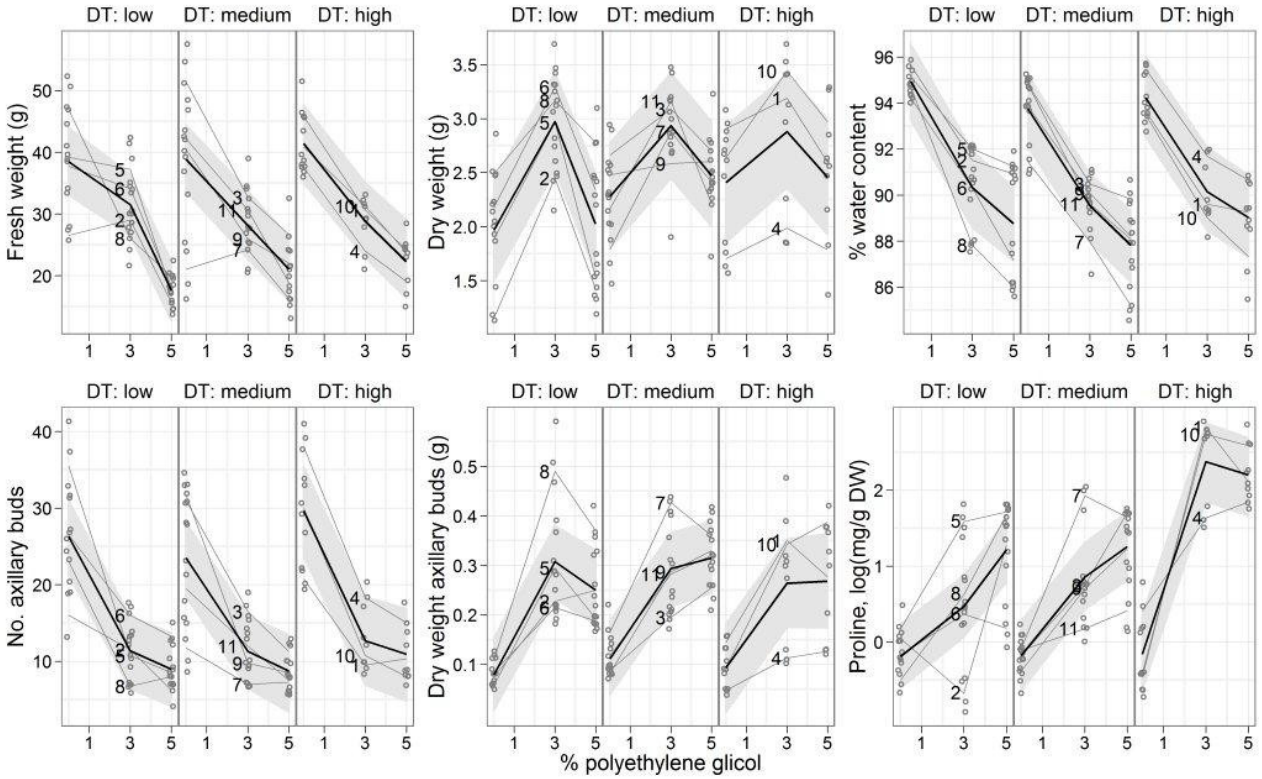

Figure 2. Effects of drought stress on growth traits and proline production of tissue-culture explants of sugar beet genotypes (1 to 11) from three classes of visually field-assessed drought tolerance (DT) cultured *in vitro* for one month at increasing concentration of polyethylene glycol (PEG). Observed values of four replicates (circles, four explants each), average genotype positions (numbered gray lines) and mean class responses with 95% confidence interval (gray banded black line).

Water content of tissues also decreased linearly with increasing PEG concentration, showing an average fall of 6 percentage points for 5% PEG, with little average difference among tolerance groups and larger differences between genotypes within groups: genotypes 8, 7 and 10 showed the largest water loss in the low, medium and high tolerance groups, respectively.

The number of axillary buds was reduced at a higher rate with PEG increase up to 3% than with the further increase to 5%; genotypes differed more in the baseline number than in the trend and the averages of the three tolerance groups did not differ noticeably. The dry weight of axillary buds increased with PEG increase up to the 3% rate, but no further. The three genotypes with the largest loss of water content also had the smallest number and the highest dry weight of axillary buds in the respective groups.

Proline production increased with PEG increase, on average exponentially for the low and medium tolerance groups, up to the 3% rate for the high tolerance group. The overall average increase at 5% PEG was fourfold for the low and medium tolerance groups, but eleven fold for the high group. Genotypes differed within groups for both baseline level and trend, with some genotypes of the low (8) and medium tolerance group (3, 9) showing proline increases comparable to those of genotypes of the high group. Proline production appeared positively correlated more with the axillary bud matter production, concentrated in fewer buds, than with the total biomass.

The results of *in vitro* culture on PEG medium seem to support the hypothesis that high accumulation of proline can mark genotypes that better tolerate stress, given the classes of drought tolerance assigned to this sample of genotypes by assessment of visual symptoms in field cultivation, though it did not discriminate for these classes in the greenhouse trial. The *in vitro* results seem to

agree with the conclusion reached by Koskeroglu and Tuna (2010) and by Roy et al. (2009), who concluded that high proline content was a good index for moisture stress resistance in rice genotypes.

In wheat, proline content increased progressively with time of stress exposure and faster rate of proline accumulation was observed in tolerant genotype than in susceptible one (Nayyar and Walia, 2003). According to results of Koskeroglu and Tuna (2010), inhibition of plant growth was not significantly affected by PEG – induced water stress in maize. Proline content increased in the leaves of maize plants grown at water stress compared to the unstressed control plants, which is consistent with results obtained in this experiment.

Comparison of greenhouse and in vitro water stress effects

Effect sizes of the stress treatment for dry weight of the plant material were negative for all genotypes in soil, ranging between -0.2 and -1.5 standard deviations, while being positive in vitro for nine genotypes, except 5 and 1 (Figure 3). Differences among genotypes were also somewhat larger in soil. Some genotypes showed good parallelism: 2 and 5 in the low tolerance group; 7, 9 and 11 in the medium group. Divergences were highest among genotypes of the high tolerance group.

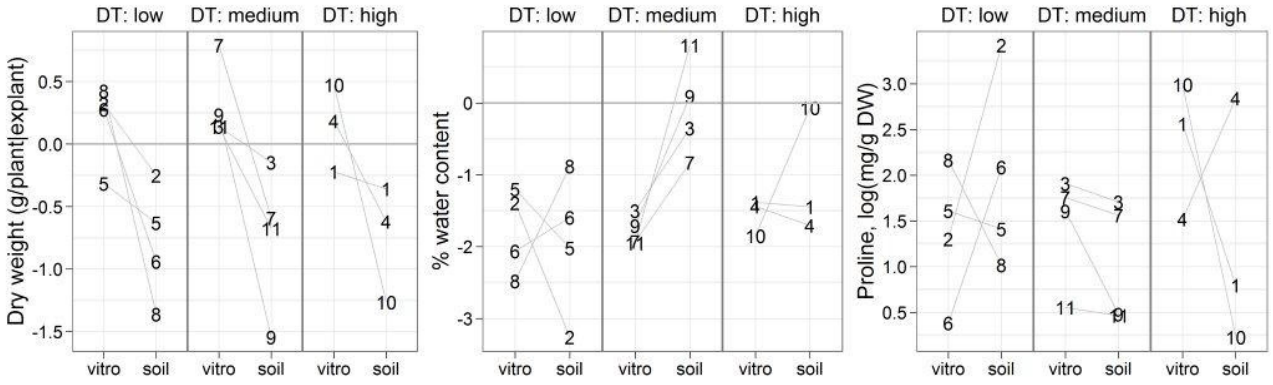

Figure 3. Comparison of effect sizes (in standard deviation units) between *in vitro* and in soil/sand mix (soil) induced drought stress on plant dry weight and proline production of sugar beet genotypes (1 to 11, connected by grey lines) from three classes of visually field-assessed drought tolerance (DT). The *in vitro* stress treatment is the highest level of polyethylene glycol (PEG) concentration (5%) in the growth medium.

Water content effect sizes were negative both in soil and *in vitro* for the low and high tolerance groups, negative *in vitro* and positive in soil for the intermediate group. Genotypes diverged more where effects were both negative: 2 and 5 with lower effects in soil against 6 and 8 with the reverse, in the low group; 1 and 4 with comparable effects against 10 with effect only *in vitro*, in the high group.

Effect sizes for proline production were all positive and in comparable ranges, in spite of substantial absolute differences between *in vitro* (six fold) and in soil (sixteen fold). Parallelism was found for some genotypes in the medium tolerance group (3, 7, 11), but divergences were considerable among genotypes in the low and high groups: 2 and 6 with larger effects in soil against 8 with the reverse in the low group; 1 and 10 with larger effects *in vitro* against 4 with the reverse in the high group. All these divergences, not matching among dry matter, water content and proline, show that the two approaches at characterizing sugar beet genotypes for water stress response cannot be surrogate of each other. While the *in vitro* proline results allowed separation of genotypes classed at the highest level of water stress tolerance in visual field assessment, those of the greenhouse test were inconclusive in this regard, maybe for having applied a temporally limited, unique, stress event instead of a continuous or intermittent lower level of water supply. Also, the largest proline effect there (sixteen fold against six fold of the *in vitro* experiment) could have masked differences which could have been detected with a milder stress level.

CONCLUSIONS

Both the *in vitro* test with increasing levels of PEG and the suspension of water supply in the greenhouse experiment showed large increases of free proline in tissues of sugar beet explants or leaves consequent to water restriction, as well as reduction in fresh weight, tissue water content and axillary bud formation. Stress effects varied considerably among genotypes classed at low, medium and high levels of field tolerance to drought stress, but were similar as class averages, except for proline *in vitro*, which was significantly higher for genotypes in the high tolerance group. Proline production was higher in the *in vitro* experiment compared with the greenhouse experiment, but the effect of PEG exposure was less strong than the effect of water stress in the greenhouse, which could have triggered a maximum response by all genotypes.

Proline production in reaction to water deficit as modulated in the *in vitro* test with PEG, appears to allow a degree of separation between sugar beet genotypes with respect to tolerance of drought as assessed visually in field cultivation.

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