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# Sugar Beet Tolerance to Drought: Physiological and Molecular Aspects

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Additional information is available at the end of the chapter

## Abstract

Drought often reduces sugar beet yield in the Balkan agroecological region. Climate forecasts indicate that this negative trend of drought periods will continue. Tolerance to drought is a complex trait, which comprises involvement of both physiological and molecular mechanisms in plants. This research was conducted on 11 sugar beet genotypes, which showed different tolerance to drought in the field. Experiment had three parts: water deficiency caused by cessation of watering conducted in the greenhouse, water deficiency imposed by different concentrations of polyethylene glycol on plants grown in tissue culture, and analysis of alterations in gene expression under drought. Plants exposed to stress in greenhouse had on average three leaves less, 4% lower water content, and seven-fold higher proline content. Classification of genotypes with respect to the level of tolerance to water deficiency on the basis of concentration of free proline, assessed in the experiment *in vitro*, corresponded to the result of the observation test in the field. Changes in the expression of candidate genes under drought suggest that one of them might be used for further development as a DNA-based marker. These results can be applied in sugar beet breeding aimed at increasing tolerance to water deficiency.

**Keywords:** water deficiency, *Beta vulgaris*, drought tolerance, polyethylene glycol, chloroplast pigments, chlorophyll fluorescence, free proline, green house, tissue culture, candidate genes

## 1. Introduction

### 1.1. Amount and distribution of precipitation required for sugar beet development

Required amount of precipitation for successful production of sugar beet is 600 mm per year [1]. Furthermore, during the winter period, sugar beet requires around 230 mm and

during the period of vegetation (from April to October) approximately 370 mm of precipitate. However, based on perennial average yield data, sugar beet production may achieve high outcome even in the presence of lower (500 mm per year) or higher (1000 mm per year) amount of rain. Water requirement of plant, during the period of vegetation, depends on precipitation. The water loss due to evaporation is most intensive from June to August when the temperatures are high and the air is dry. The average potential evapotranspiration (ET) for period of 30 years in case of sugar beet is 576 mm, but it may vary between 528 and 625 mm due to weather conditions. Approximately 10–20% of total water requirement of sugar beet is fulfilled from the soil water reserves and the rest is obtained by precipitation and irrigation. The amount of water lost by transpiration is 392 mm in average, and it varies from 198 mm during dry years to 542 mm during rainy years. The average precipitation during vegetation (April–September) is 359 mm and it varies from 138 to 521 mm in certain years [2].

Having in mind the above-mentioned facts, amount and distribution of precipitation, in combination with the light and amount of heat, mostly determine quality and yield of sugar beet. On the territory of Serbia, it is common that the lack of soil water, typical for summer months, sometimes occurs during moderately rained years. Lack of soil moisture outcomes 100–200 mm per year, but rarely exceeds 300 mm per year. Currently, less than 1% of irrigation-suitable agricultural land in Serbia is intensively irrigated.

Climate of Serbia is continental or moderate continental. The most important sugar beet production area is Vojvodina region situated in the north of the country. Climate of Vojvodina is moderated continental, determined by the presence of Alps on western border of Pannonian basin, Carpathian Mountains, the Dinarides, and the Balkan Mountains [3]. Precipitation regime is continental, typical for Danube region, with precipitation maximum in summer (June) and minimum in winter. According to the Köppen classification [4], for the period of 1961–1990, dominant climate type in Vojvodina is *Cfwbx″* [*C* = mild temperate climate; *f* = significant precipitation during all seasons; *w* = dry winters; *b* = warmest month averaging below 22°C (but with at least 4 months averaging above 10°C) and *x″* = second precipitation maximum occurs in autumn] [5].

Brief analysis of current and expected precipitation distribution during winter, spring, and sugar beet growing season in Vojvodina is made using the data from two meteorological stations located in southern and northwestern part of the Vojvodina region, Novi Sad (Rimski Sancevi) and Sombor, respectively. Climatological data for 1971–2000 refers to the climatological periods derived from the database of the Republic Hydrometeorological Service of Serbia (RHMSS). Future climatic conditions were obtained from the Eta Belgrade University (EBU)—Princeton Ocean Model (POM) model for the A1B scenario for 2001–2030 and A2 for 2071–2100 integration periods [6]. Obtained results lead to expected shift in climate types from *Cfwbx* to "*Cfwax*" in the prevailing part of the country indicating temperature of the warmest month above 22°C (letter *a* in the Köppen formula) [5].

Overview of the average precipitation for the selected past climatological period indicates that during winter time of 1971–2000 reference period, the amount of precipitation is twice less than optimum ones, while growing period of precipitation was slightly below optimal values.

According to climate model simulations for 2001–2030 integration periods, expected average annual precipitation during first decades of the twentieth century, at selected locations, will not vary significantly in relation to the 1971–2000 precipitation records (**Table 1**). Inspection of the precipitation amounts for 2001–2014 period (**Table 2**) witnesses in favor to this expectation, with 699.8 mm in Novi Sad and 668.0 mm in Sombor in comparison with the expected 2001–2030 average (**Table 1**). However, it is important to notice significant variability of precipitation in this period which is in accordance with climate simulations for 2001–2030 [3]. In regards to winter and spring precipitations, for all integration periods, climate model simulates slightly higher average precipitation in comparison to reference climatology. Less optimistic is the expectation that growing period precipitation supposed to decrease towards end of the century, with particularly vulnerable summer period and increasing variability.

## 1.2. The impact of water deficiency on sugar beet production

Water shortage during vegetation is a frequent and a significant issue in agricultural production. Possible solution to this problem is selection of genotypes, which do not show decreased yield under economically acceptable level, in the presence of water shortage. Great challenge in the process of genotype selection is to choose the convenient plant idiotype for the present agroecological conditions. Water deficiency has complex impact on plant physiology. First indicators of water deficiency in plants are the loss of turgor pressure and stomatal closure [7]. Photosynthesis is also highly dependable on the plant's water supply. Many studies showed that disruption of water flow causes decrease in water content in assimilation tissue, which leads to photosynthetic depression [8]. Based on this, soil moisture, as well as relative air humidity, determines photosynthetic intensity. A decrease in chloroplast size, an increase

| Climatol. period | Annual | Winter (DJF) | | Spring (MAM) | | Growing season | |
|---|---|---|---|---|---|---|---|
| | Precipitation (mm) | Precipitation (mm) | Variability coeff. (%) | Precipitation (mm) | Variability coeff. (%) | Precipitation (mm) | Variability coeff. (%) |
| | Novi Sad (Rimski Sancevi) | | | | | | |
| 1971–2000 | 604.1 | 108.1 | 46.2 | 146.6 | 33.3 | 359.2 | 28.8 |
| 2001–2030 | 641.8 | 131.3 | 56.1 | 159.7 | 34.7 | 369.2 | 26.1 |
| 2071–2100 | 560.5 | 131.0 | 54.5 | 147.4 | 37.0 | 282.2 | 32.0 |
| | Sombor | | | | | | |
| 1971–2000 | 580.3 | 107.6 | 41.9 | 133.3 | 29.6 | 339.4 | 22.7 |
| 2001–2030 | 629.4 | 127.3 | 56.7 | 144.6 | 37.2 | 356.5 | 29.0 |
| 2071–2100 | 565.8 | 127.2 | 53.6 | 148.1 | 41.3 | 277.8 | 31.9 |

**Table 1.** Past (1971–2000) and future (2001–2030 and 2071–2100) climate precipitation data for Novi Sad and Sombor locations in Serbia.

| Year | Novi Sad (Rimski Sancevi) | Sombor |
|---|---|---|
| 2000 | 252 | 231 |
| 2001 | 929 | 749 |
| 2002 | 412 | 448 |
| 2003 | 491 | 434 |
| 2004 | 797 | 816 |
| 2005 | 726 | 753 |
| 2006 | 640 | 585 |
| 2007 | 754 | 683 |
| 2008 | 539 | 598 |
| 2009 | 617 | 615 |
| 2010 | 1035 | 994 |
| 2011 | 382 | 401 |
| 2012 | 480 | 446 |
| 2013 | 723 | 692 |
| 2014 | 989 | 932 |
| Average | 679.8 | 653.3 |

**Table 2.** Annual amount of precipitation for 2001–2014 in Novi Sad (Rimski Sancevi) and Sombor locations.

in stomatal density, and disruption of thylakoid membrane structure were reported as consequences of water deficit [9]. Besides decrease in tissue water content, water shortage may cause synthesis of specific compounds in the roots, during the early growth phase. According to this, roots are very significant sensors of soil changes (not only in terms of water, but also texture changes), which alert the aboveground tissues by "chemical drought signals" which are transported to leaves. These signals mostly refer to plant hormones such as abscisic acid (ABA) [10].

### 1.3. Sugar beet tolerance to water deficiency

Adaptation of plant metabolism on stress conditions is species specific and was the subject of numerous studies [11]. Plants more tolerant to drought have longer root system with bigger absorptive area, better developed photosynthetic parenchyma, thicker cuticle, smaller leaf area (LA) and number of stomata per leaf area, and higher density of conductive elements [12]. They also possess highly expandable protoplasm, higher content of bound water and osmolytes, enhanced accumulation of ABA, free proline, and alanine. The following indicators point out to higher phenotypic tolerance of sugar beet to water shortage: more shiny leaves, higher turgor pressure of petiole, and more sensitive leaves to expansion [12]. Even though there is genotypic variability with respect to response to drought in sugar beet (i.e., [13]), structural and morphological mechanisms still remain unclear.

Stress occurrence during early stages of growth and development may adversely affect sugar beet root growth, which may result in yield loss by 46% [14]. In addition, later stress occurrence may cause decreased leaf area and also number of leaves and by that, the efficiency in light usage becomes decreased [15]. Water deficiency significantly increases concentrations of potassium and sodium, which disturb sugar extraction from roots. Plant response to water stress can partially be explained by disorders in mineral nutrition. Water deficiency actually may retard or even stop ion assimilation, which results in perturbation in ion ratios in specific tissues. This trend is manifested through ion deficiency symptoms in plants. The adverse effect of water stress in later phenophases is less pronounced, since plants already developed root system and canopy which completely covers the soil. Well-developed root system increases efficiency in water extraction and usage, which results in higher tolerance to water deficiency [16]. However, first signs of water stress are usually seen on leaves. Minor drop in leaf water potential may cause significant decrease of total leaf area, and the low water potential enhances emergence of new leaves and accelerates senescence of old leaves. Drought stress results in stomatal closure, limits the transpiration which increases leaf temperature [15]. Both, lower stomatal density and heat stress decrease photosynthetic outcome [17]. Sugar beet leaves have higher number of smaller stomata on their abaxial side. Higher density and smaller size of stomata is a form of adaptation to drought, because it allows plants to be more efficient in regulation of water transport and transpiration [18]. Varieties more efficient in tolerating lack of water are proven to have decreased stomatal density (70–150 stomata/mm$^2$) [19]. During drought, when negative turgor pressure in guard cells generates, small epidermal cells with tightened cell walls increase plant resistance towards water stress [20]. Response of sugar beet genotypes to drought may also be affected by percentage of adaxial and abaxial epidermis and palisade tissue thickness [21].

## 1.4. Chemical response of sugar beet to drought stress

Plants also osmotically adapt to drought [22]. Exposure to water deficiency results in accumulation of osmolytes, such as betaine, proline, and fructans. These substances often accumulate in the form of compatible solutes in plants (compounds which do not take part in chemical reactions in plants, but affect cell water potential), which generate expression of genes encoding relevant enzymes. Osmolyte production, as well as change in osmotic pressure, may increase sugar beet tolerance to abiotic stress. Proline and glycine betaine help in the preservation of cell [23], which makes them suitable for further investigation with purpose of increase stress tolerance of many species including sugar beet [24, 25]. They are not only involved in maintenance of cell turgor and osmotic balance but also in protection of cell structure from stress [26]. However, it still remains unclear whether the plants, which accumulate osmolytes, better tolerate lack of water or not [27].

## 1.5. Proline accumulation

Free proline is a key metabolite which accumulates in sugar beet exposed to drought [28]. Change of the free proline concentrations in tissues is an indicator of other kinds of stress, such as temperature, environmental pollution, and misbalanced nutrition. The same factors may affect

glucose accumulation and yield. In some cases, stress conditions may increase sugar beet root quality and potential of recovery if plants were not highly damaged by water deficiency [29].

Higher nitrogen supply increases proline content and may also increase leaf area index (LAI) and drought stress impact. Positive and significant correlation among proline and glucose content in sugar beet root indicates the relationship between the response to stress, carbohydrates, and proline and glucose accumulation ratio. This is supported by the effect of treatment with di-1-p-mentene (anti-transpirant) and DMDP (2,5-dihydroxymetil-3,4-dihydroxypyrolidine, glycosidase inhibitor), which decreased proline content in roots of irrigated sugar beet [29]. Presence of compounds such as proline and glucose adversely affect sugar crystallization and lead to the formation of colored components, thus reducing industrial quality of beet roots [30].

Proline accumulated in sugar beet root, as a nitrogen compound, reduces the quality of roots. Both, the stress and an excess of nitrogen lead to the mobilization of accumulated carbohydrates, which are the source of energy essential for adaptation to the stress conditions. Moreover, chemicals containing nitrogen (e.g., proline) reduce the yield of sucrose and the quality of the roots [29]. The importance of the accumulation of proline in osmotic adjustment is still debatable and varies from species to species [31]. The highest proline accumulation was observed at the end of beet root growth [29]. Correlation between drought and proline content suggests, however, that alteration in proline concentration is useful stress indicator in sugar beet [28]. Proline may act as a signal molecule which alters mitochondrial function, affects cell division and gene expression. This role of proline may be very significant for plant recovery when favorable conditions are regained [32].

### 1.6. The use of plant biotechnology to increase tolerance to water deficiency

Basic need for sustainable food production directed research programs towards improving traits of crops despite the size and complexity of their genome [33]. Plant biotechnology is a process in which the use of molecular and cytological techniques help to increase the productivity of the plants, to improve the quality of plant products, to prevent the damage caused under the influence of various biotic and abiotic stresses. Plant breeding reliving on the employment of molecular markers [Marker Assisted Selection (MAS)] is one of the promising techniques to improve crop resilience. A prerequisite for the success of MAS is defining the genes which regulate traits of interest and to test relationships between potential markers and those traits. Only when this link is defined, i.e., when the marker is physically located in the vicinity of or even within the gene of interest, it is possible to use it efficiently in breeding [34].

In sugar beet, development of breeding programs aimed to increase drought tolerance is further complicated by the fact that several types of abiotic stresses often occur at the same time during the growing season, and approach which involves a manipulation of a group of genes for tolerance to drought seems necessary to solve this complex problem [35].

In an era of rapid progress in the identification and characterization of complete segments of plant genome, proteins, transcripts, metabolites, as well as their interactions in a biological system, new discoveries will lead to better understanding and possibly to manipulation of physiological responses to water deficit [36]. Evaluation of the relative contribution of genes

conferring tolerance to the dehydration and elimination of those which do not affect the tolerance to stress is a major challenge.

Although the yield is the basic goal of the breeders, it is very difficult to accurately predict the possibility of water utilization and identify candidate genes for further cloning [37]. Several studies have identified quantitative trait loci (QTLs) associated with a specific component of the response to drought. Although the development of molecular markers and genome sequencing should expedite positional cloning [38], genome areas associated with individual QTLs are still very large and usually not suitable for testing in the breeding program. With the rapid development of genomic technology and the suitable statistical methods, there is an increased interest in the use of mapping strategies for the identification of genes encoding quantitative traits which have agricultural or evolutionary significance [11]. Another major challenge is how to apply knowledge to improve crop tolerance to stress conditions. There is a problem between high yield and tolerance to stress since very often genotypes with higher stress tolerance have lower yield under optimal conditions. One of the strategies for sugar beet phenotyping was proposed by Ries et al. [39].

On the cellular level plant adaptation to stress includes regulation of the beginning of protein synthesis (e.g., $H^+$ pumps and $Na^+/H^+$ antiporter), an increase in antioxidant level, transient increase of the concentrations of ABA, the reduction of the energy consumption ways, as well as accumulation of the solution, and protective protein [40]. All of these changes at the cellular level are of great importance for the maintenance of homeostasis after ion imbalance caused by abiotic stress [26]. The deficit of water causes the synthesis and accumulation of ABA in plant cells and the genes corresponding to this has been defined. Most of these genes contain conserved cis-activating promoter elements, called Abre (ABA-responsive element, PyACGTGG/TC) [41]. Great progress to clarify the response of plants to abiotic stress has been made in the last decade [11].

In order to achieve a combination of high yield and tolerance to stress in one variety, it is necessary to establish a connection of development of individual characteristics and mutual reactions, which can be achieved only through co-operation among molecular biologists, physiologists, and breeders [11, 42]. It is necessary to assess the relationship between different morphological, anatomical, physiological, and biochemical traits of sugar beet tissues in different phases of their growth and development during different periods of water shortage, in order to categorize genotypes with respect to their tolerance to drought which was in the focus of our previous [21] and present study.

## 2. Material and methods

### 2.1. Plant material

The study involved 11 genotypes (marked from 1 to 11) of sugar beet (*Beta vulgaris* ssp. *vulgaris*, L.) differing in levels of drought tolerance, according to observation test conducted in the field. According to this test, genotypes were divided into three groups: (1) sensitive genotypes: 2, 5, 6, and 8; (2) moderately tolerant: 3, 7, 9, and 11; and (3) tolerant: 1, 4, and 10.

Experiment was conducted in three stages:

1. Under semi-controlled conditions in greenhouse.

2. In in vitro conditions of tissue culture.

3. Gene expression analyses of water regime responsible genes in leaves (plants from the greenhouse experiment).

### 2.1.1. Experiment under semi-controlled conditions in greenhouse

Sugar beet seeds were sown in growth substrate Potgrond H (Klasmann), mixed with river sand (17.5:1) in plastic pots (31 × 37 × 13 cm). A single pot contained 12 plants. During 90-day period, soil moisture was kept at 80% field capacity. Plant watering was conducted on the basis of evapotranspiration. When the plants were at the 6–12 leaves stage, they were exposed to water deficit by cessation of watering, while control plants were watered. Five days later, molecular and physiological analyses were done.

After drying plant material on 105–130°C to its constant mass, % of dry matter was determined. Activity of photosynthetic apparatus was assessed by monitoring of $F_{0(initial)}$, $F_{m(maximal)}$, $F_{v(variable)}$, $F_v/F_m$, and $t_{1/2}$ using plant stress meter (PSM, BioMonitor S.C.I. AB). Free proline concentration was measured in the both *in vitro* and *in vivo* conditions [43]. Concentration of chloroplast pigments was determined spectrophotometrically [44, 45]. Leaf area (LA) was measured by automatic leaf area meter LI-3000 (LI-COR, USA).

### 2.1.2. Experiment in tissue culture

In this experiment, MS basic substrate was used [46] with 0.3 mg/l BA (benzyldenine) and 0.01 mg/l $GA_3$ (gibberellic acid). In order to obtain sufficient number of axillary shoots (64), equal in size, subcultivation was done every 3 weeks. Lack of water was caused by addition of polyethylene glycol to the substrate. Obtained shoots were set on a substrate for micropropagation with 0, 3, and 5% of polyethylene glycol (PEG 6000, Duchefa, Netherlands). Plants were cultivated on this substrate for 4 weeks and afterwards fresh weight of shoots, as well as dry matter and free proline content were determined. The temperature during the experiment in air conditioning chamber was 21–23°C, with a photoperiod of 16 h of light and 8 h of dark.

### 2.1.3. Gene expression analyses of water regime responsible genes in leaves (plants grew in the first experiment)

The changes in gene expression were analyzed in the leaves of the sugar beet plants grown in the greenhouse experiment. Candidate genes were selected from the previous studies [47–50]. For 13 candidate genes which are, considered to be, involved in osmotic and salt stress responses, primers were constructed and used to screen for polymorphisms at the DNA and gene expression levels. Ten selected candidate genes were homologous probes (BI543470, BI096135, AW697770, BI543640, BG932913, BI096146, BQ060651, BF011094, BI096078, and BF011254), and heterologous probes from maize (X15290), alfalfa (BI543243), and carrot (BI073246). Samples

for DNA/RNA analysis (leaves) were taken 5 days after the last watering (experiment 1) and used for DNA/mRNA extractions. mRNA was used to synthesize cDNA, and this cDNA was template in further PCR reactions [42].

## 2.2. Statistical analyses

Statistical analyses of data were performed by different statistical methods. ANOVA was applied, to photosynthetic pigments (MCMCglmm Methods, [51]), using Package R (http://www.jstatsoft.org/v33/i02/). Logarithmic and Jonson's transformations (Minitab) were performed for parameters with large data variability, in order to normalize their 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 [52] and the contributed packages lme4 [53] and ggplot2 [54].

# 3. Results and discussion

As previously indicated (**Tables 1** and **2**), climatic conditions in our region suggest the need for research, which has the potential to enhance selection of genotypes more tolerant to drought.

## 3.1. Experiment under semi-controlled conditions in greenhouse

### 3.1.1. Sugar beet genotype classification based on physiological tests in semi-controlled conditions

Sugar beet genotypes in semi-controlled conditions showed different reactions to 5-day water deficiency. As expected, decline in turgor was observed in all genotypes. Number of leaves was significantly different between treatments and respective controls. Concentrations of photosynthetic pigments and leaf area varied between genotypes and standard normal distribution was not observed here. Therefore, the data were subjected to Johnson's data transformation which proved to be very effective [55]. This procedure allowed assessing differences in concentrations of photosynthetic pigments between different genotypes (**Figure 1**).

Secession in water supply caused water loss from plant tissues within both sensitive and tolerant genotypes. Due to this fact, sugar beet genotypes may be divided on the basis of tested parameters and following treatments (**Figure 2**).

The results obtained in semi-controlled conditions (experiment 1) were compared to previous field observations (**Figure 3**). Proline concentration increased in all genotypes after exposure to water deficit as well as % of DM (except for genotypes 9 and 11). Changes within treatments with respect to control, referring to dry weight were less pronounced than changes referring to % of DM and RWC of root, stem, and leaf. Plants subjected to stress conditions had in average three leaves less, 4% higher % of DM, and seven times higher proline content.

The relationships between two effects on measured traits were assessed by mixed model (**Figure 4**). Crossed pink lines in diagrams represent average impact on genotypes in control
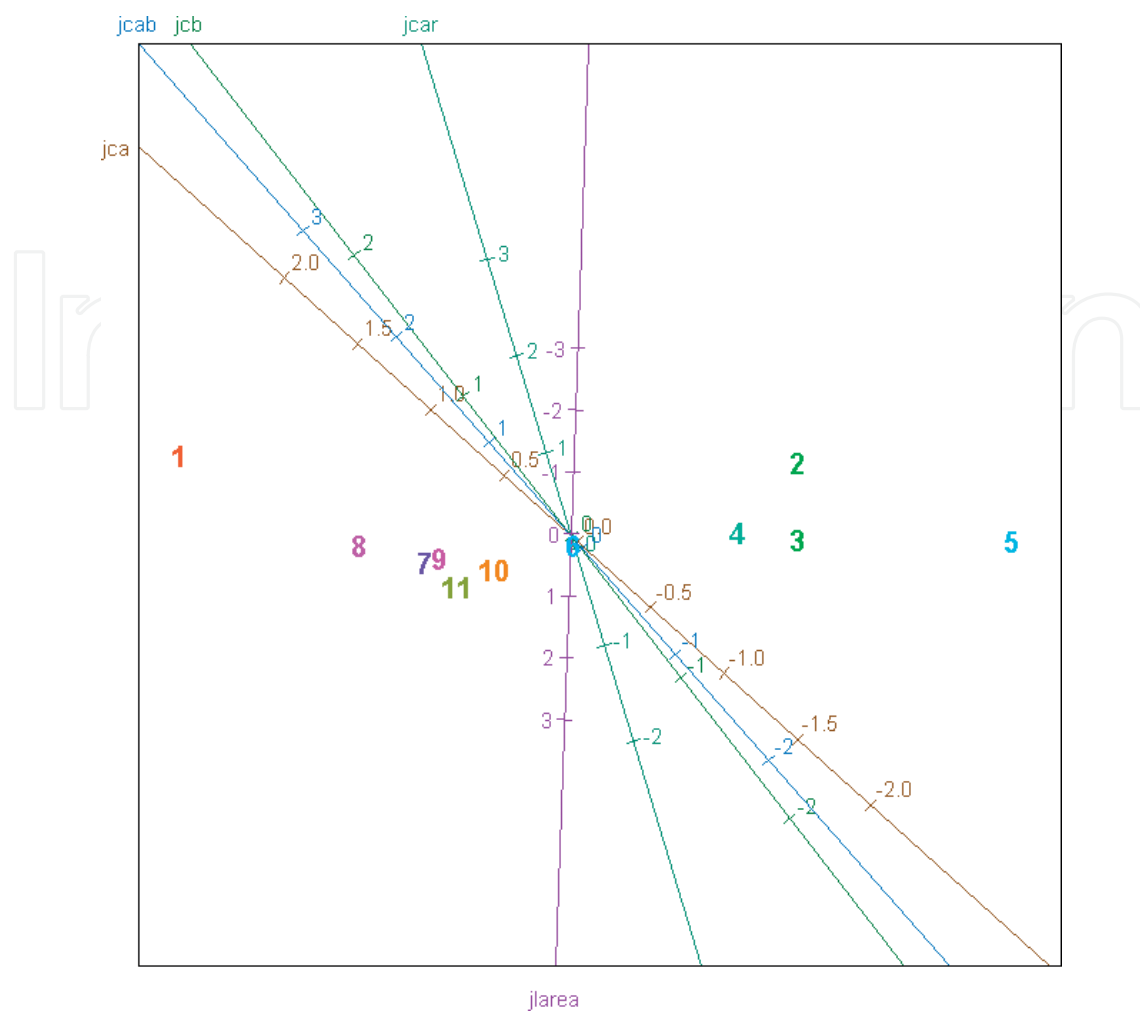
**Figure 1.** Genotype separation on the basis of pigment concentration and leaf area for variables normalized according to Johnson's data transformation (jlarea—leaf area; jcar—carotenoids; jca—chlorophyll a; jcb—chlorophyll b; jcab—chlorophyll a + b).

(x axis) and stress effect (y axis). There is a nearly perfect negative correlation between the unstressed value and the response to stress for root DM and a similar, but weaker one, for leaf number. Genotypes showing positive scores for the slope effect (y axis) are less affected than the average by (more tolerant to) water stress for the involved trait, and vice-versa. Genotypes showing positive scores for both effects are both higher scoring in absence of stress and less affected than the average by (more tolerant to) stress for the involved trait, and vice-versa.

Conventionally, results of chlorophyll fluorescence indicate a high sensitivity to influence of ecological factors. Therefore, it is often used as an indicator of functioning of photosystem II.

According to $F_v/F_m$, sugar beet genotypes were compared on the basis of photosynthetic characteristics. Water deficit did not cause significant variations in fluorescence indicators (**Figure 5**).
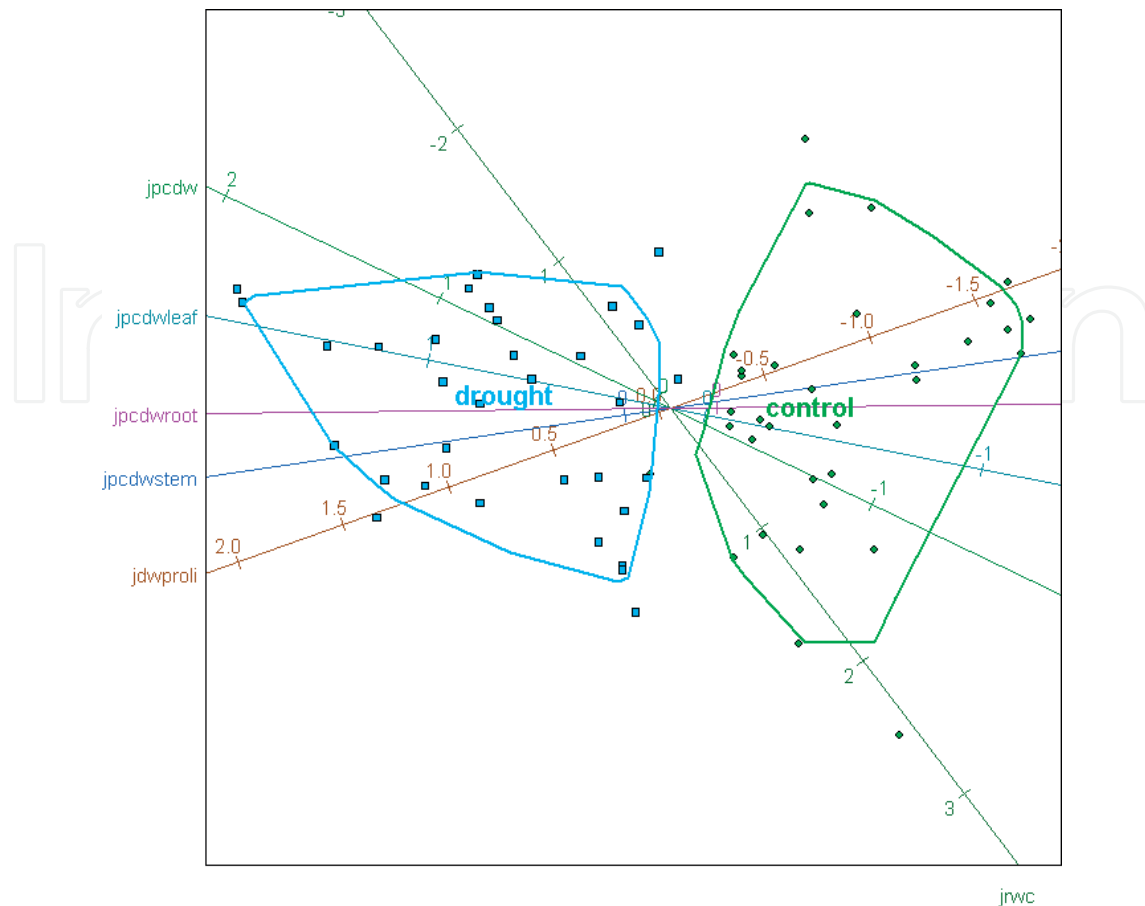
**Figure 2.** The separation of the sugar beet genotypes based on experiments in greenhouse with a highlighting treatment (control, drought) for a variable normalized by Johnson's transformation (jrwc—relative water content; jpcdw—dry weight; jdwproli—free proline; jpcdwleaf—leaf dry matter; jpcdwstem—stem dry matter; jpcdwroot—root dry matter).

Effects of drought were observed in case of $F_v$ and $F_m$, but not for $F_v/F_m$ ratio, where the largest differences between genotypes were obtained. In addition, overlap of intervals of interaction between stress and genotype indicates stress, which caused differences, similar for all genotypes. The influence of water deficiency on fluorescence may be related to plant tolerance towards water deficit in field conditions (**Figure 5**).

Plant development may be inhibited in different ways in field conditions. It may be affected by interactions among drought and other ecological stresses, precipitation, and temperature availability as well as interactions with different micro-organisms [36]. On the contrary, semi-controlled conditions may only eliminate interference of other factors with plant development. Therefore, it is necessary to compare results obtained in the greenhouse with those obtained in the field.

### 3.2. Experiment in tissue culture (*in vitro*)

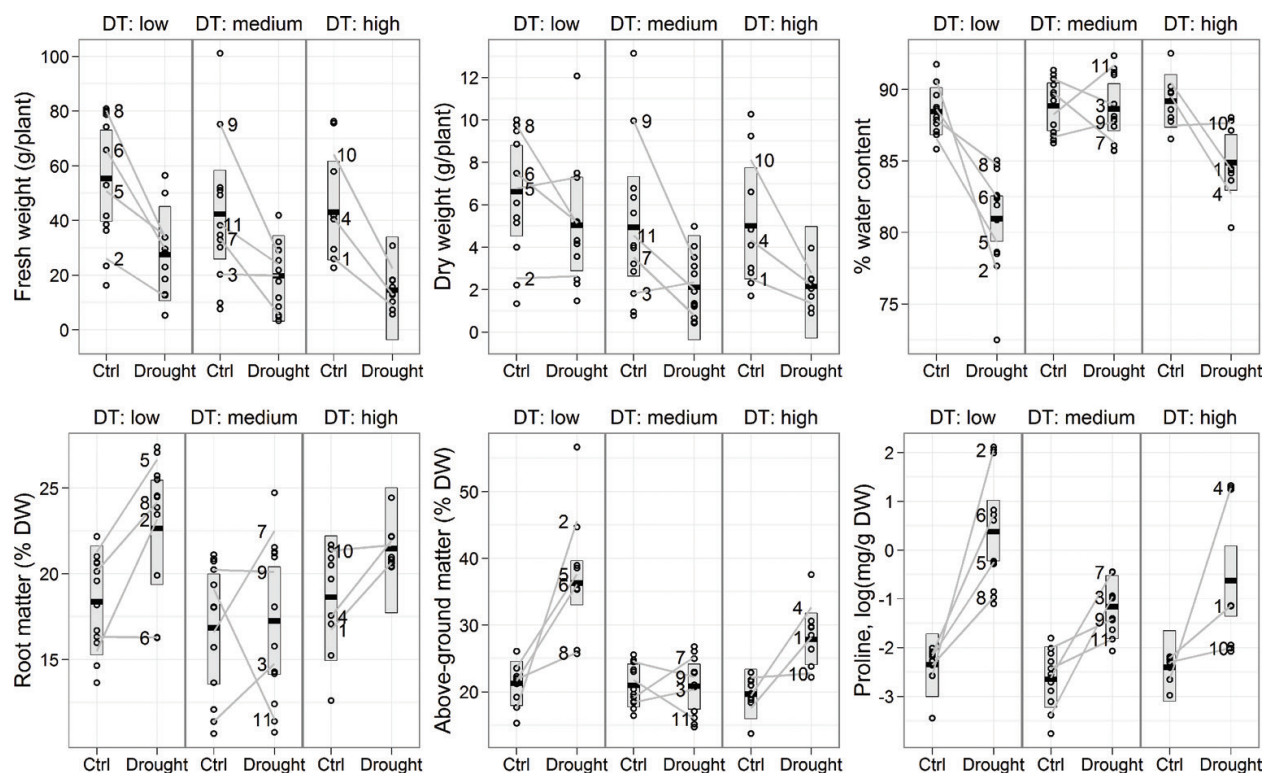Increased PEG concentration decreased growth of axillary buds with respect to control (**Figure 6**).

**Figure 3.** Effects of drought stress on growth traits and proline production of greenhouse grown plants of sugar beet genotypes (1–11) from three classes of visually field-assessed drought tolerance (DT). Observed values of three replicates (circles, 10 plants each), average genotype positions (numbered gray lines), and class means with 95% confidence intervals (crossbars). The drought stress was induced by suspension of watering to test plots after 3 months of culture and observations were made after 5 more days [28].

Number of axillary shoots may be indicator of the influence of different PEG concentrations, which cause water deficit, on micropropagation potential of genotypes. Average number of axillary shoots of 11 subjected genotypes showed 2.2 times decreased number of shoots in the presence of 3% PEG and 2.7 times in the presence of 5% of PEG.

The degree of tolerance to drought observed in the field corresponded to tolerance recorded in the experiments performed in the greenhouse and in tissue culture (**Figure 6**). The most prominent criterion for estimation of genotype tolerance to drought was found to be concentration of free proline [28]. Proline concentration was significantly increased in leaves exposed to drought and axillary buds and it was positively correlated with PEG concentration, which is in accordance with the results of other researches [56].

PEG treatment decreased total dry weight and number of axillary shoots by more than twice, while presence of 3% PEG in the substrate increased total fresh weight. Furthermore, PEG caused decrease in water content in tissues and decreased number of buds, but increased bud weight and % of DM. The highest values were recorded in control (0% PEG) for total fresh weight, in the presence of 3% PEG for proline concentration and fresh weight of axillary buds and in presence of 5% PEG for % of DM. Fresh weight of plants grown in presence of 3 and 5% PEG decreased (**Figure 6**). Average dry weight of the plants was the highest in the presence of 3% PEG. However, in the presence of 5% PEG, it was almost in line with the control. Higher
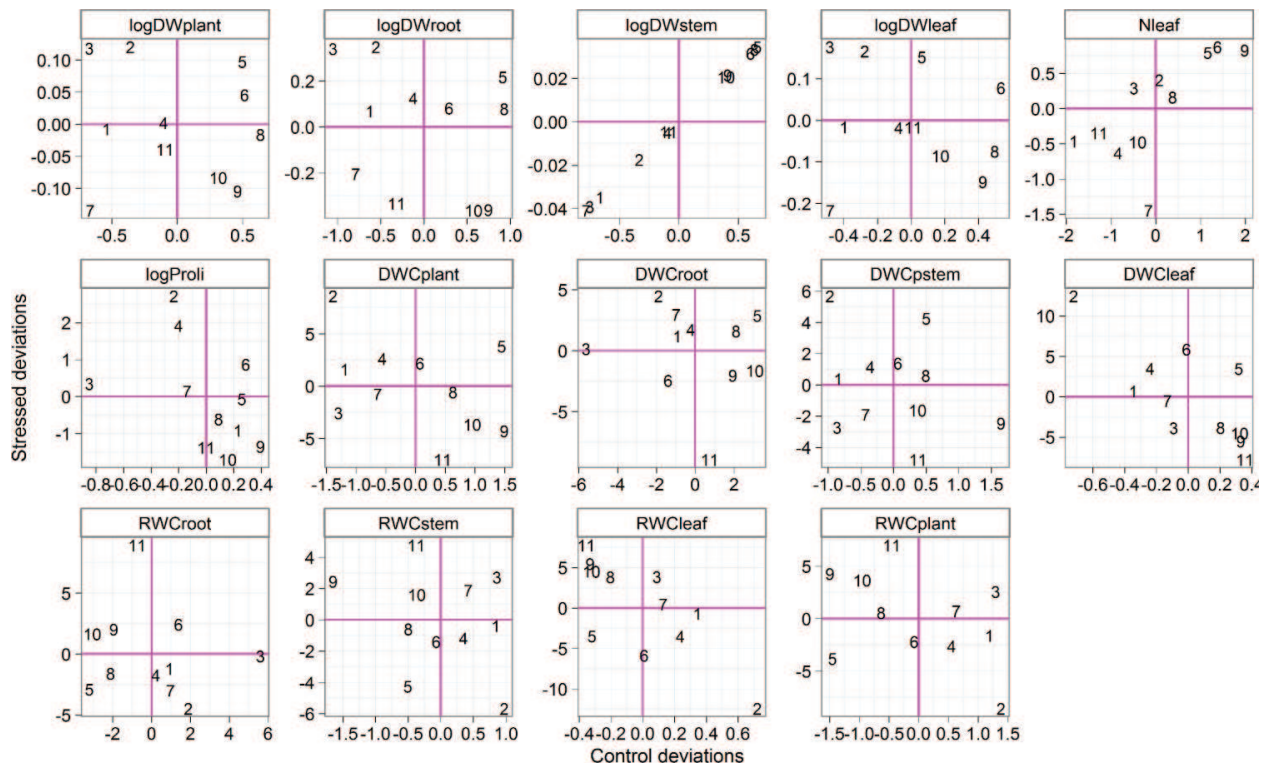
**Figure 4.** Genotype effects from mixed model analyses for traits of greenhouse experiment (deviations for values in unstressed condition on the x axis, deviations for stress effects on the y axis. Genotypes are identified by the numbers).

variability in dry weight was recorded in the group of drought sensitive group (according to field observations), but the same trend as in the other two groups of genotypes remained. Tissue water content linearly decreased following the increase in PEG concentration, the average drop in presence of 5% PEG was 6%, and was followed by the low average difference among groups of different tolerance and higher difference among genotypes of one group (**Figure 6**).

Proline accumulation under stress conditions increased under treatments in both experiments. In tissue culture, it was 6 times increased and in greenhouse 16 times with respect to corresponding controls.

If taking into account the genotypes tolerance in the field, in relation to the parameters obtained from the analysis of plants in tissue culture and in experiment in the greenhouse, dry matter, in relation to the water content and the concentration of proline is not significantly different among groups of the tolerance (**Figure 7**).

Recorded differences between genotypes show that there are two approaches for the separation of sugar beet genotypes in relation to response to water stress, which cannot substitute each other. On one hand, proline content in plants grown in tissue culture enabled to match their grouping with respect to observations in the field. On the other hand, experiment in greenhouse was less efficient in that sense (**Figure 3**). The main cause of this may be the fact that stress in the field was not continuous as it was in the greenhouse.
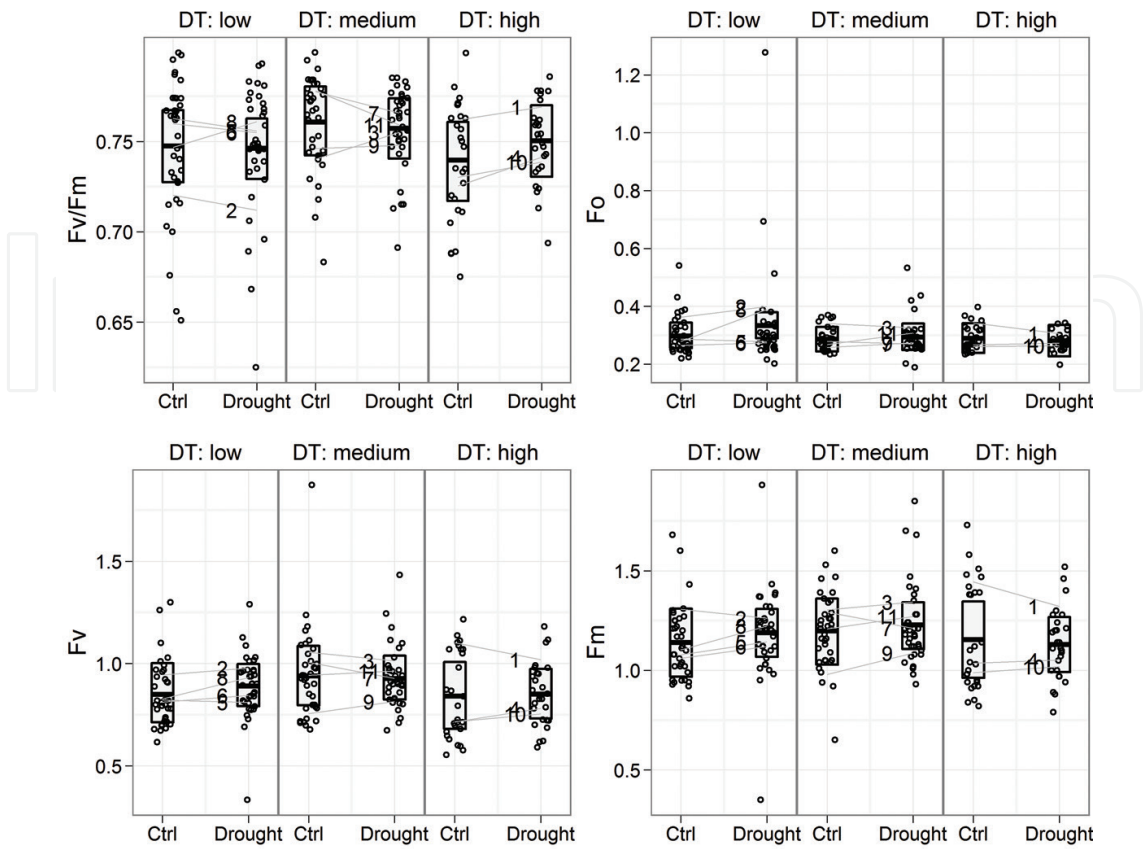
**Figure 5.** Maximal ($F_m$) and variable ($F_v$) chlorophyll fluorescence and their ratio ($F_v/F_m$) in sugar beet genotypes grouped according to their field-assessed drought tolerance (ctrl—control; drought; DT—drought tolerance).
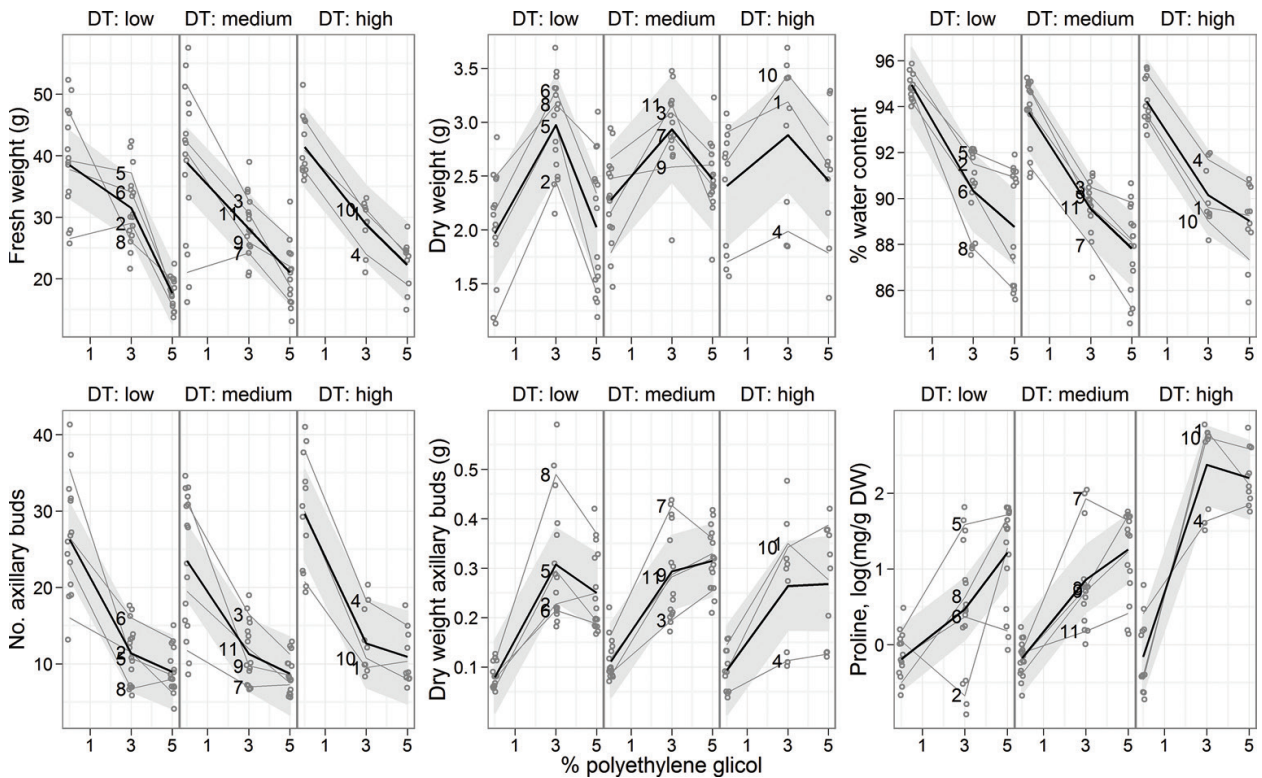


**Figure 6.** PEG effect on growth traits and free proline concentration of plants cultivated in tissue culture [28].
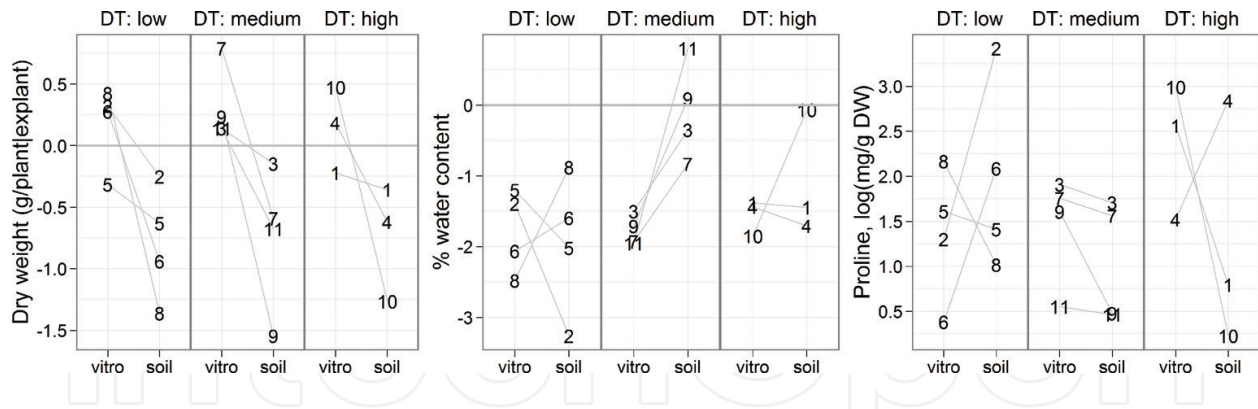
**Figure 7.** Water deficit effect on dry weight, water content, and free proline concentration in greenhouse and in tissue culture experiment [28].
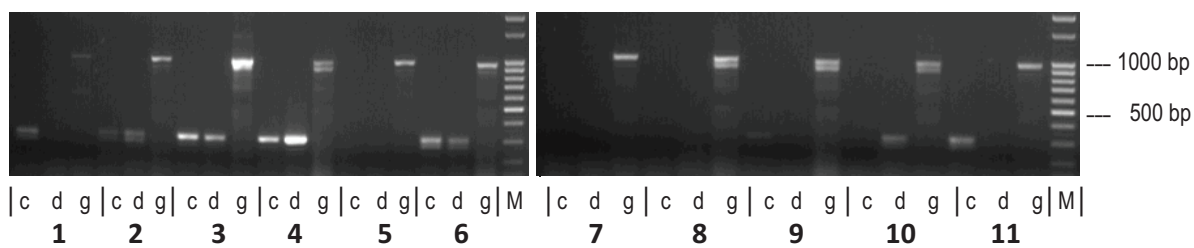


**Figure 8.** Expression pattern of gene corresponding to BI543243 in sugar beet leaves (c—template cDNA deriving from control plants; d—template cDNA deriving from plants exposed to drought). Amplification on genomic DNA served as additional control (g). M—100 bp DNA ladder size marker.

### 3.3. Analyses of changes in expression of genes involved in reactions to water stress (plants from greenhouse experiment)

Changes in the expression of 13 candidate genes in 11 different sugar beet genotypes were followed in leaves of plants grown in the greenhouse. Expression pattern corresponding to BI543243 differed in plants exposed to drought in comparison with corresponding controls in genotypes 1, 10, and 11 (**Figure 8**). Therefore, it may serve to develop molecular marker useful to differentiate genotypes with respect to drought.

## 4. Conclusion

Tolerance to drought is very complex. Experiments in three different environments (tissue culture, greenhouse, and field) with 11 genotypes, where many different parameters were followed, revealed that it is not easy to find single criteria for classification with respect to drought tolerance. However, the results suggest that free proline accumulation may be used as a reliable parameter. The classification based on changes in concentration of free proline in plants exposed to drought in greenhouse and tissue culture corresponded to classification made on the bases on field observations. Therefore, similar fast tests, conducted with young plants and possibly aided by the use of molecular markers, can be useful for estimation of breeding material with respect to tolerance to water deficiency, which will significantly enhance sugar beet breeding for expected future changes in climate.

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