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A METHOD FOR THE IMITATION OF DROUGHT STRESS IN ARABIDOPSIS THALIANA L.

A method for the imitation of drought stress in Arabidopsis thaliana L. combining the advantages of rapid and prolonged treatments was developed. The proposed method allows decreasing water content in the growth medium gradually and homogenously, growing plants under sterile conditions, and observing a root system during the growth. In the course of experiment, gradual growth retardation of stressed plants was observed. The rates of cell division and cell elongation of the tap root were measured and it was defined that cell division and cell elongation are both responsible for growth retardation. Morphological changes in the roots of stressed plants were described.

Plant growth is greatly affected by environmental abiotic stresses such as drought, high salinity and low temperature. These stresses induce various biochemical and physiological responses in plants to acquire stress tolerance. Abiotic stresses are severe limiting factors of plant growth and crop production [31, 22, 23]. Among these abiotic stresses, drought or water deficit is the most severe limiting factor of plant growth and crop production [22, 11]. It causes 24 million tons of yield loss in maize annually by inhibiting plant growth and photosynthesis [25]. Typically, however, roots are affected less then shoots. In fact, even under mild water deficit, shoots may stop growing completely while roots continue to grow. Continued root growth allows plant to plumb soil for water and can be especially important for seedling establishment [31].

Although the responses of plants to water deficit have been studied in many species, we thought to use *Arabidopsis thaliana L.* to take advantage of the potent molecular and genetic tools available for this species [31, 35]. Its many advantages include a small genome, short life cycle, small stature, prolific seed production, and ease of transformation. In addition, a wealth of genomic resources exists, such as a completely sequenced genome, a near saturation insertion mutant collection, a genome array that contains the entire transcriptome, and more then 50,000 molecular markers [35]. In addition, roots of *A. thaliana* seedlings have a well-defined anatomy [31].

In the last years a number of methods have been worked out, which allow imitating conditions of natural drought stress. These treatments could be divided into two big groups: prolonged [30] and rapid treatments [9, 26, 34]. In the case of prolonged treatments, stress action lasts from several days to several weeks. Plants are grown on sand or soil, and desiccation occurs through ceasing of watering. Stress conditions are more similar to those of natural water stress. Another advantage is the possibility to observe long-term morphological changes. At the same time difficulties are possible by reliable determination of the humidity of sand or soil and hence it may be problematic to estimate the stage of stress. Root system can't be observed *in vivo*, and it could be complicated to isolate whole plants including roots for analysis. As a result of using of non-sterile growing substrate, sterility is not maintained.

Taking into account disadvantages of prolonged treatments, another group of methods has been established and widely used. We called them rapid treatments because stress

action doesn't exceed several hours. Desiccation occurs through removing of plants from growth medium and putting them on filter paper under controlled temperature and humidity. These methods are brief and reliable; whole plants can easily be separated from medium and taken for biochemical as well as for microscopic analysis. At the same time, they are very simplified imitation of natural water stress; duration of stress doesn't allow observing long-term morphological changes.

Despite the advantages of *A. thaliana*, to our knowledge only few attempts were undertaken to imitate conditions of prolonged water stress on plants growing on sterile agar medium [26]. Unfortunately, our attempts to repeat these experiments resulted in unequal drying of medium and contaminations. Nutrient-agar media are often used in studies of *A. thaliana*, and hence a large amount of physiological data has been obtained for these conditions [31]. Therefore, the aim of this study was to develop a method to grow *A. thaliana* seedlings at a lowering water potential on an agar-solidified medium; and, using this system, we characterized several growth responses of *A. thaliana* seedlings to water deficit.

Materials & Methods

Plant Material

Arabidopsis thaliana L. line used in this study was Columbia wild type. The CycB1; 1: CDB: :GUS marker line was kindly provided by John Celenza.

Plant Growth Conditions

A. thaliana seeds were sterilized in 5% NaClO for 3 minutes, washed 3 times in sterile water, and distributed in 1% low melt agarose. Afterwards the seeds were sown onto the standard plastic agar plates containing 1x Murashige and Skoog (MS) salt mixture supplemented with 4.5% sugar, pH 5.7, and left to imbibe 2 days at 4˚ in the dark. The plants were then cultivated in a growth chamber in a near vertical position at 22 ˚C, and 16-h light (80 μmol $m^{-2}s^{-1}$) /8h dark cycle till the age of 6 days which corresponds to the stage of fully developed cotyledons. Then, the plants were transferred onto plates with low MS concentration (0.25x) and without sugar. Sucrose, often compared to a hormone, was added to the medium because it promotes plant growth [1, 31]. In the experiments with the medium lacking sugar, germination and growth were irregular to such an extent that it was difficult to select enough uniform seedlings for transplanting [31]. At the same time, to minimise the consequences of osmotic stress, we used a second medium without sugar and with the low concentration of mineral nutrients (0,25x MS).

Additionally, the plates intended for the modulation of water stress conditions were modified. Firstly, the amount of medium was fixed to 80 ml. Then, the medium was poured under determined angle so that the thickness of agar layer on the top of the plate is less as on its bottom, and hence the drying gradient is created. Finally, the standard plate plastic cover was replaced with cellophane foil (Roth, Germany), which is transparent for water vapour. Control plates were standard i.e. they contained 0.25x MS 40 ml each and were covered with usual plastic cover. Plants were viable under these conditions till the age of 5—6 days after transfer.

Measurement of Substrate Moisture

The small pieces of agar substrate were taken from the surface of angle plates, which were placed into growth chamber, at the level of 2, 6, and 10 cm from the top of the plate. Then, the agar pieces were dried for 4 h at 70 ˚C, and the dry weight was calculated.

GUS-Staining

The mitotic activity of A.thaliana roots was investigated by using the cycB1;1:CDB:GUS marker line. The plants were removed from agar every day after transfer, submerged in staining solution (0.1 M $NaPO_4$ pH 7.0, 0.01 M EDTA, 0.01% (v/v) Triton X-100, 0.5 mM K_3[Fe(CN)6], 0.5 mM K4[Fe(CN)6] and 0.05% (w/v) X-Gluc substrate solved in dimethylformamide). Samples were mounted on micro-scopic slides and incubated at 37 ˚C for 2 hours. The number of dividing cells in the

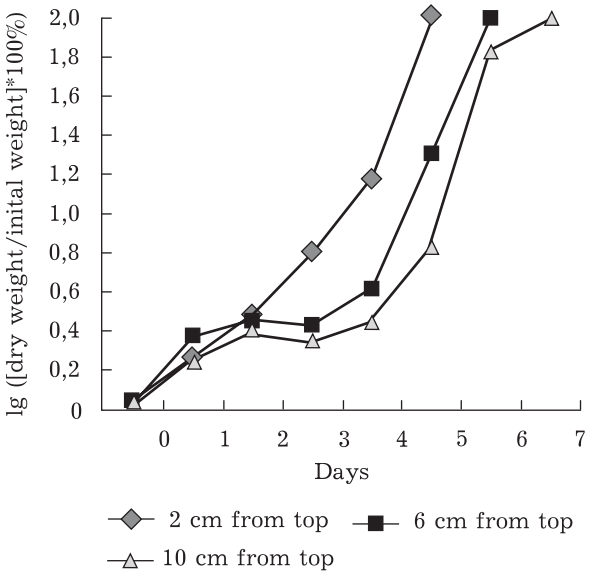

Fig. 1. Change of substrate humidity of an angle plate

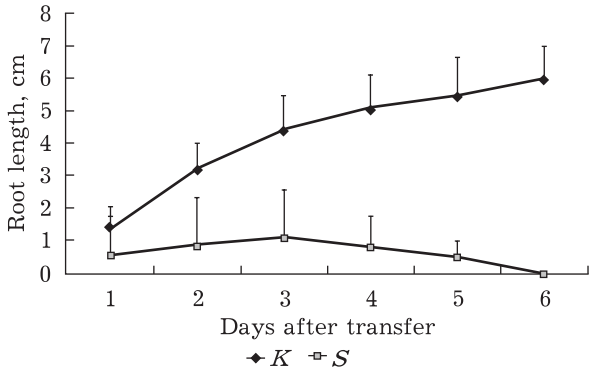

Fig. 2. Change of length of main root of plants grown under stress conditions and in control. *Symbols indicate*: *K* — control plants; *S* — plants subjected to slow desiccation stress. Bars indicate standard deviations

meristematic zone of the main root was counted under the light microscope.

Imaging of Roots and Cell Length Measurement

Images of roots of growing plants were captured with a Leica DC500 digital camera. The control and stressed plants were harvested at the age of 5 days after transfer, fixed in a mixture of methanol and acetic acid (3:1, v/v). The samples were mounted on the microscopic slides in mounting solution (76% (w/v) chloralhydrate, 19% (v/v) glycerine 87%). For the measurement the fully elongated epidermal cells were taken, which do not form root hairs (atrichoblasts) along the entire main root, starting at 2mm from the distal end of the main root and finishing at the point where transfer occured. This point could be easily seen by the abscence of the root hairs, which were lost at the moment of transfer. The cells were measured using micrometric ocular (10×). One randomly selected cell within an interval of 0,5 mm was measured. The measurements were taken using a Zeiss Axiowert 35 microscope.

Results and Discussion

System for observation of growth of A. thaliana plants upon slow desiccation stress

Arabidopsis thaliana plants grown till the age of 6 days on the full MS medium supplemented with 4.5 % sugar under standard conditions were subjected to slow desiccation stress by transferring on the plates specially constructed for this purpose. We measured the dry weight of the substrate at 2, 6 and 10 cm from the top of the angle plate (Fig. 1). This resulted in a progressive depletion of water in the substrate. From the onset of the drought conditions (0 d), the dry weight of substrate increased from the initial value of 1,1% to 100% (no water) within 5, 6 and 7 days at the level of 2, 6 and 10 cm from the top of the plate, respectively. The character of curve testifies the loss of approximately equal portions of water day after day.

As a result of water loss, the stressed plants were visually much more retarded in comparison to control. To gain further insight into the response of primary root elongation to water deficit, the kinetics of the response during the 5 d exposure was studied. As shown on Fig. 2, in the control plants, soon after transfer, we observed the subsequent activation of growth, return to the log-phase, and reaching of the plateau. In contrast, the stressed plants have undergone after the period of slight activation on 2 d and 3 d the phase of slow subsequent retardation of growth followed by death on 6 d as a consequence of total water loss. Under

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stress conditions, the total elongation of the main root at the end of the 5 d treatment was approx. 6 times less as in the control.

Most studies have found that water deficit inhibits primary root elongation rate [10, 13, 16, 27, 31]. Stimulation has been shown for *Pinus pinaster* [18, 29], *Glycine max* (L.) Merr. [5], and *Arabidopsis thaliana* [31]. In our experiment, the decrease of primary root growth rate is more pronounced at the later stages of stress, which is in accordance with an observation that at moderate stress the root elongation increases whereas at severe water stress it decreases [31]. As described above, the desiccation stress results in a progressive retardation of root growth. It is well known that adverse conditions inhibit root growth and that cell division and cell cycle regulation are involved in this response [3, 12, 20, 21, 33]. To investigate whether this phenomenon is a result of more rare division of meristematic cells or it reflects the lower level of cell elongation, we undertook two experiments.

In course of the first one (Fig. 3) *A. thaliana* plants (ecotype Columbia) were fixed on the 5 d and observed in light microscope. We measured the length of mature epidermal cells, which do not produce root hairs, so called atrichoblasts. Cells were measured only in the part of the main root, which had grown after the transfer. This "post-transfer" distal part of the root could easily be distinguished from the "pre-transfer" one by the presence of root hairs in it. At first we expected the change of a cell length corresponding to the phase of growth, e.g. longer cells at the beginning of stress action and shorter at its end. Nevertheless, no considerable change of cell length within two groups could be observed, though elongation rates changed considerably in both groups. Cell length in the control increased quite slightly compared with stressed plants. At the same time, this difference was not significant, and it could not be responsible for much more strong elongation of a main root of control plants. Therefore, a length of epidermal cells seems to be parameter, which should be regulated to fall within a preferred range.

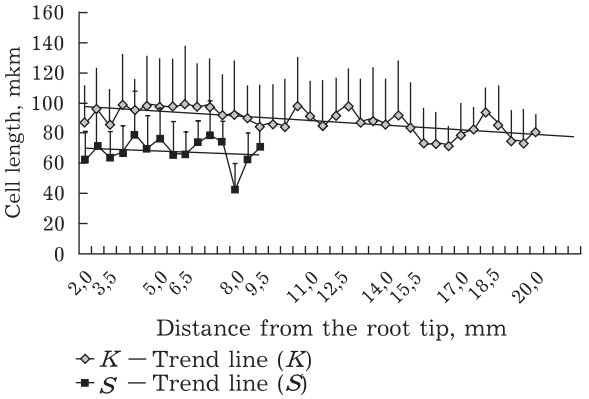

Fig. 3. Length of mature root epidermal cells in control and under slow desiccation stress. *Symbols indicate*: K — control plants; S— plants subjected to slow desiccation stress. Bars indicate standard deviations

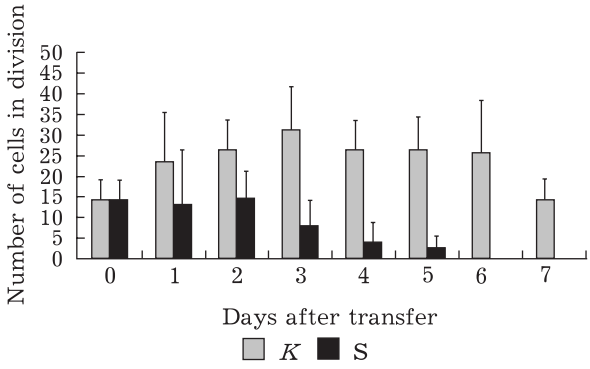

Fig. 4. Number of dividing cells in the root meristem of plants grown under stress conditions and in control. *Symbols indicate*: *K*— control plants; *S* — plants subjected to slow desiccation stress. Bars indicate standard deviations

This allows us to presume that the changes in division of root meristematic cells are responsible for changes in root growth rate and hence for the reaction of plants to the conditions of the slow desiccation stress. In order to check this hypothesis, we investigated the mitotic activity using the plants of a transgenic marker line Cyc B1;1: CDB: GUS, which were expressing the labile translational fusion protein between the mitotic cyclin B1 and the β-glucuronidase. The promoter and the region coding the first 150 amino acids of *A. thaliana* cyclin B1 were combined with the

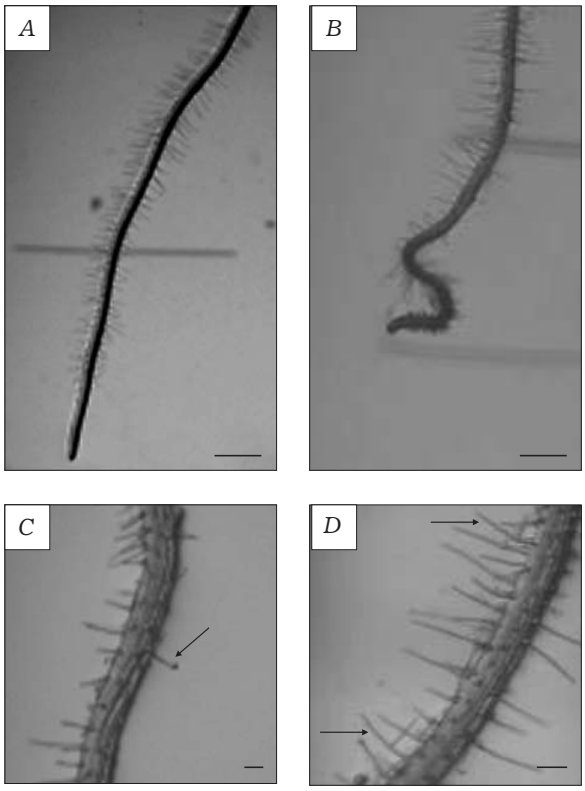

Fig. 5. Root phenotypes. Bars: 1 mm in *A* and *B* and 100 μm in *C* and *D*.
A — Control plant. Regular distribution of root hairs could be observed; *B*—*D* — plants grown under stress conditions; *B* — bending of root tip. Root hairs in the area of tip bending have peculiar shape, which may be result of turgor loss in the distal part of the root. Irregular distribution of root hairs and hairless areas could also be observed; *C* — root hair with vesicular extension at its distal end (indicated by Arrow); *D* — root hair branching. Arrows indicate position of root hairs with bifurcations

β-glucuronidase (GUS) gene. The expressed chimeric protein contains the destruction box (CDB), which resides between amino acid 30 and 38 of cyclin B1. Owing to a mitotic degradation signal in the protein, reporter gene activity marks only actively dividing cells [4, 15, 32]. The plants were daily taken from agar, stained, and the number of stained cells was measured (Fig. 4). Parallel we carried out the measurement of root elongation of transgenic plants, which was similar to the root elongation of Columbia wild type plants (not shown). In the control group, the division of meristematic cells of main root changed over time in parallel with an elongation rate. The same goes for the group of stressed plants with one noteworthy exception: division rates on 3 d, 4 d and 5 d were much higher as it could be expected from their elongation rates. This may be due to a fact that root elongation is always a result of *previous* cell divisions.

To our knowledge, in *A. thaliana*, the change in root elongation rate is paralleled by the change in cell production rate. Our data provide another support for this hypothesis. In species where water deficit similarly inhibited primary root elongation rate, cell production rate was similarly inhibited [6, 8]. This suggests that the root's elongation rate at given levels of water deficit is determined principally by the supply of cells to the zone of rapid elongation [2, 31].

The most sensitive cells of a root might be root hairs. They display a tendency to a big morphological plasticity. Under slow desiccation stress, the roots exhibited hair decay (Fig. 5B), which was stronger under more severe stress conditions. Typical for the root hairs of stressed plants are also such abnormalities, as root hairs with vesicular extension on their distal end and root hairs with bifurcations (Fig. 5C, D). To our knowledge, this is the first report of the enhanced production of root hair branching under conditions of water deficit. Bifurcated root hairs is one of the numerous root hair deformations, which is promoted by several treatments, such as influence of Nod factors (bacteria-to-plant signalling) [7] and Fe-deficiency [17]. This response to signal occurs at a specific developmental stage, namely when hairs are terminating growth [14]. At the final stages of the slow desiccation stress, the roots exhibit curling of their distal end (Fig. 5B). The similar phenomenon was already observed [28] in the experiments with gel drying. This may be an indication that the drying of gel surface increases its sticking capacity, which in its turn is capable of slowing the movement of

root tip, and causing deflections as a result of elongation. In association with curling the "crumpled" root hairs could be also observed, which may be due to lower turgid pressure at the distal end of the root.

In this work we presented a new method of slow desiccation stress, which combines the advantages of rapid [9, 26, 34] and prolonged [30] treatments. This method allowed us to observe gradual retardation of growth and other effects of drought stress action (bifurcated root hairs, root tip bending, root hair loss).

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Recommended to publication by B.O. Levenko

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МЕТОД ІМІТАЦІЇ ВОДНОГО СТРЕСУ НА ARABIDOPSIS THALIANA L.

Автором розроблено метод з імітації водного стресу на проростках Arabidopsis thaliana L., який дає змогу знижувати вміст води в середовищі поступово і рівномірно, вирощувати рослини в стерильних умовах і вивчати кореневу систему під час росту. В процесі експерименту відмічене поступове пригнічення росту рослин в умовах водного стресу. Встановлено, що поділ та елонгація клітин є відповідальними за пригнічення росту рослин порівняно з контролем. Описано морфологічні зміни в коренях рослин в умовах водного стресу.

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МЕТОД ИМИТАЦИИ ВОДНОГО СТРЕССА НА ARABIDOPSIS THALIANA L.

Автором разработан метод имитации водного стресса на проростках Arabidopsis thaliana L., позволяющий снижать содержание воды в среде медленно и равномерно, выращивать растения в стерильных условиях и изучать корневую систему во время роста. В процессе эксперимента отмечено постепенное угнетение роста растений в условиях водного стресса. Установлено, что деление и удлинение клеток являются ответственными за угнетение роста растений по сравнению с контролем. Описаны морфологические изменения в корнях растений в условиях водного стресса.