**Final report of OTKA grant 76273 on**

**The role of root development in the drought adaptation of cereals**

## Introduction

As early as in the middle of the XX century we had to learn that plant cells are more autonomous than animal cells and plant cells maintain totipotency, a capability to return from differentiated physiological state to meristematic cell state which is the functional equivalent of the recently discovered stem cells of animals and human. This transition takes place in plant cells via reactivation of their cell cycle. In this project, we take the advantage of *Brachypodium distachyon*, a model plant for grass species including important crop cereals like wheat, barley and rye as well, to understand how this question of "to divide or to differentiate" is regulated in plant cells. Root development was chosen as the developmental process to study because a strong and efficient root system is also extremely important for the survival of our crop plants under stressful environmental conditions. Work on *Brachypodium* as model plant for temperate grasses started here in 2009.

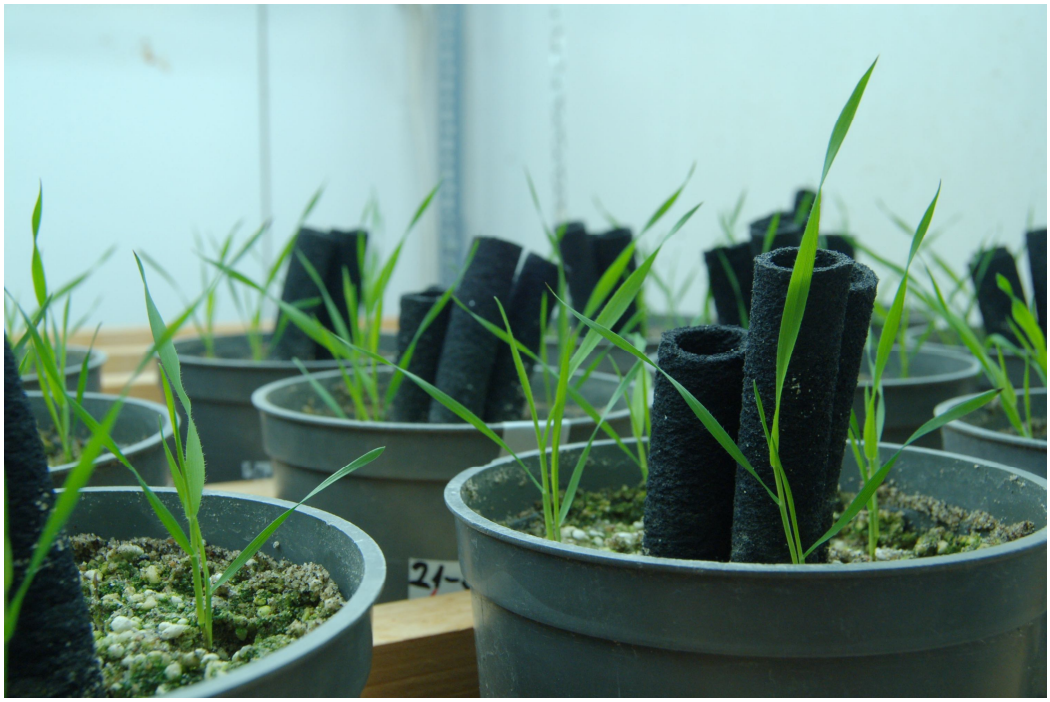


**Figure 1. Developing *Brachypodium* plantlets under controlled water regime.**

## Results

In the frame of this project, our results on the root development and transcriptional profile changes of six wheat cultivars differing in their drought adaptation strategies has already been published (Secenji et al, 2010 Plant Biology; Szécsényi et al, 2013, CRC).

To migrate to *Brachypodium*, both our previous experience on wheat and rice and the experimental system used for them, had to be applied and tested on the new model plant. Finally, similar system to the one used for wheat in recent years, was established to study the root development of *Brachypodium*. This rhizotron based system enables the continuation of research an the same question: how can grass plants reshape their root system as the consequence of limiting water supply and what are the candidate genes playing role in the changes of the root architecture that we observe on morphological level in genotypes accommodating to drought stress well.

Wide range of *Brachypodium* genotypes differing in drought tolerance was tested, selected lines were characterized in details in rhizotron in terms of root growth and architecture and general growth parameters. Main component analysis revealed that thick and deep growing primary and nodal roots are important for good long term drought adaptation.

### Characterisation of *Brachypodium* genotypes

During the first year of the project, plant growth conditions were optimized for purple false brome (*Brachypodium distachyon*) both in greenhouse and in controlled climate room. Thirty one *Brachypodium* accessions were obtained from seed collection of the *Brachypodium* research community (for complete list see Table 1 of Appendix 1). Based on various parameters of their aerial parts, these ecotypes differ in their drought response characteristics covering wide range from being very drought sensitive to drought tolerant. Extensive propagation (seed multiplication) of these lines has been started; 3-5 generations were necessary to generate sufficient amount of seeds to start physiological characterization.

Experiment were carried out using sand:perlite (1:1) or vermiculite:perlite (1:1) mixtures as soil substitutes. These mixtures allow both the RNA preparation from the collected root samples and the tracing of roots in rhizoboxes. In order to mimic natural drought conditions limited water supply was used resulting in 20-40 % water saturation. This water limitation still allows the long term adaptation and survival of the plants, however already causes growth retardation and mild visible symptoms of drought stress. Increase of abscisic acid levels in the leaves at limiting water supply also confirmed the development of stress in the plants.

Physiological characterizations of these ecotypes were performed in two experimental systems. In the pot system, more than twenty parameters were measured under control (80% water saturation) and water limiting (40% water saturation) conditions, principal component analysis revealed those parameters which are the most discriminative between both genotypes and water regimes. The first two components contained the majority (82%) of the total variance: PC1 was related to shoot height, fresh weight and dry weight as well as first leaf node axile root number; while PC2 was mainly driven by first leaf node axile root total length together with coleoptile node axile root diameter at the root tip. Hierarchical clustering of the plant lines revealed six clusters. One representative of each cluster were further investigated in rhizobox experimental setup which allowed direct observation of the root system architecture throughout the whole growth period.

Taken together, shoot length and weight are well-known parameters which demonstrate the detrimental effects of drought stress. Besides these, the principal component analysis revealed that the number and length of the leaf node axile roots and the diameter of the coleoptile node axile roots are the appropriate parameters to characterize the response of the root system of *Pooideae* plants during water limitation in the vegetative phase. Based on the cluster analysis on the data obtained from pot experiment, the six accessions that were selected for rhizobox analysis were suitable for examining different adaptation strategies to water-deficit stress. Therefore, these *Brachypodium* genotypes can serve as good candidates to investigate the genetic and gene expression background of the early response of the root system of *Pooideae* crops grown during water-limited conditions. For further details see manuscript in Appendix 1.

**Gene expression studies, LOB-domain transcription factor family**

At the beginning of the project, 15K oligonucleotide chip was designed on Agilent platform including 24 cyclin gene and 20 LOB-domain gene for which at least partial sequence information could be obtained. However, the completed genome sequence of *Brachypodium* based on the most widely studied Bd21 line was published in February, 2010, the whole gene expression work could be revised and more direct approaches could be used.

The work plan has been supplemented with *in silico* promoterom analysis. To do this, an enumeration algorithm has been developed which is capable to predict those genes which may belong to the same regulon (Cserháti et al, 2011, MGG). In the first step, the complete promoterome database of *Brachypodium* was sorted out of the annotated genome sequence. It contains the 2 kilobase 5' regulatory sequences of all the predicted genes in the genome. We looked for genes possessing root specific expression profile and sorted out those promoter elements (dyad sequences) that are common in these promoters. All together 47 genes were found which seem to be root specific, confirmed by the presence of at least 3 corresponding root specific EST-s for each of them. The comparison of the promoters of these 47 genes to a random promoter set revealed 215 dyad motives which are now suitable tools to predict the other entire root expressed genes in the *Brachypodium* genome.

As candidates of root architecture influencing genes, we study lateral organ boundary (LOB) domain transcription factor family of *Brachypodium*. Since the discovery and initial description of this gene family, wide range of knowledge has been accumulated but still the exact functions of these proteins are hardly known. Most of the data derive from *Arabidopsis* and little is known from monocots.

Analysis of the complete genome sequence resulted in 24 members of this gene family in *Brachypodium*. Based on the comparison of the most conserved regions of their coded protein sequences a similarity tree was built (Figure 2). To determine typical expression profile of these genes, members of each subgroup of the tree were analyzed using gene specific primer pairs in quantitative real-time PCR on various RNA samples.

For successful sampling and RNA purification of the expression work, a new method of sample collection had to be used. The separation of numerous root zones (tip, elongation zone, root hair zone, lateral root initiation zone etc.) is laborious and time consuming. During this time the tiny roots of *Brachypodium* taken out of the soil-substitute, start drying and the RNA content of the root starts decay making it unusable for further work. After several different sample collection attempt, quick, forced desiccation a roots gave us the solution: it keeps the RNA sufficiently intact while we have time to dissect the roots and collect the necessary few dozen mg tissue from each root part for RNA purification. A modified CTAB protocol also overcame the problem of RNA isolation from dried material.

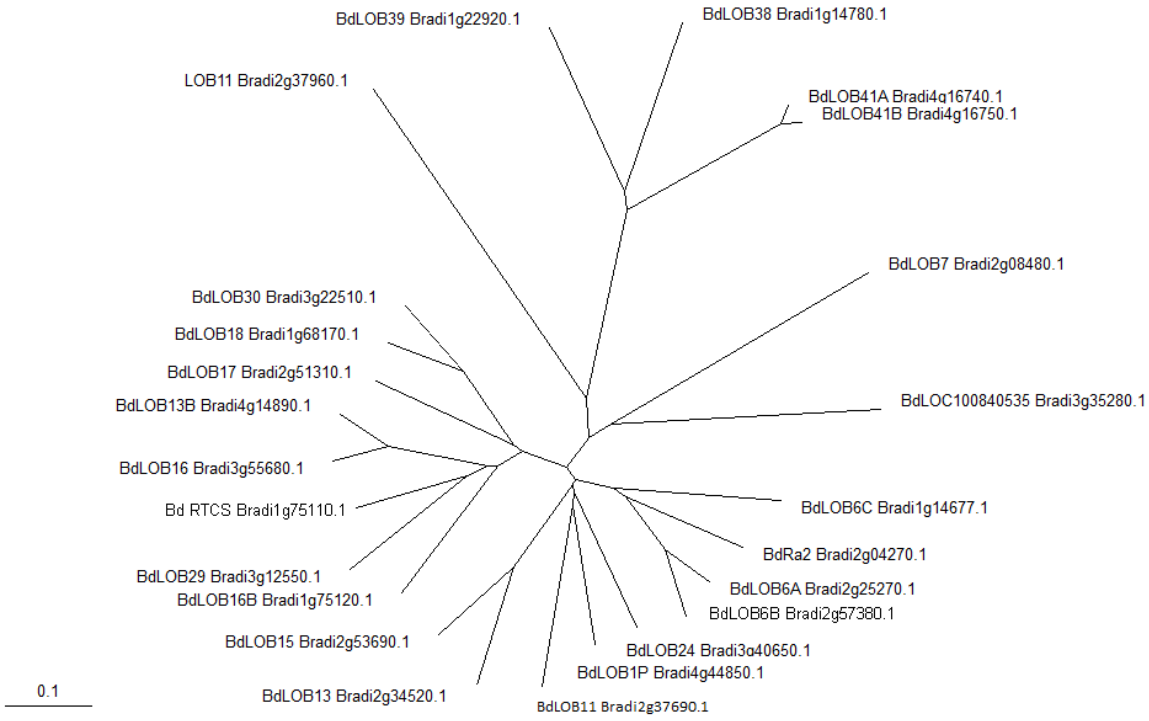



**Figure 2. Phylogenetic tree of *Brachypodium* LOB-domain transcription factor coding genes.** The tree was made upon the conserved amino acid sequences of LOB domains.

RNA was purified from 33 different plant parts ranging from root tips to developing young seeds. First strand cDNA was reverse transcribed from them and qRT-PCR

(quantitative real-time polymerase chain reaction) using gene specific primers were performed in ABI Prism 7900 RT-PCR equipment. Relative mRNA levels were determined by the 2ΔΔ-CT method using EIF-4A and UBC genes as internal references.

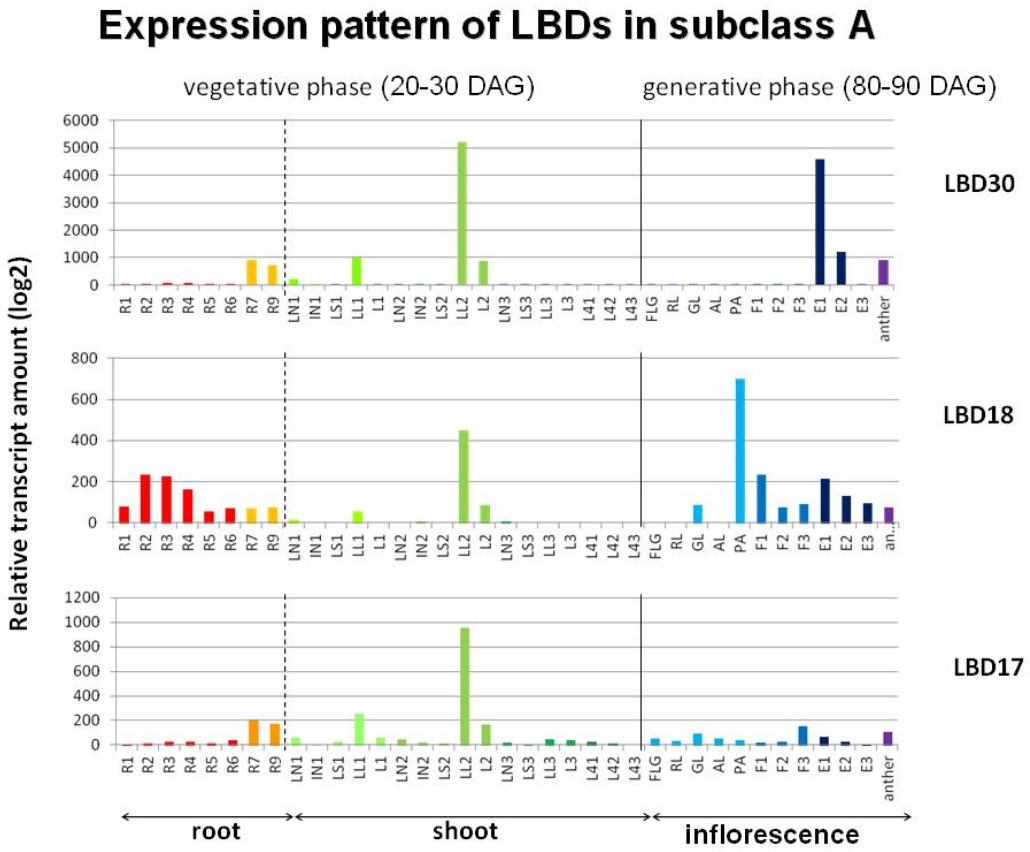
Abbreviations and color code of the samples:

| | vegetative organs |
|---|---|
| R1 | root tip |
| R2 | elongation zone |
| R3 | root hair zone |
| R4 | maturation zone |
| R5 | young lateral root node |
| R6 | old lateral root node |
| R7 | hipocotil node |
| R9 | coleoptil node |
| LN1 | node of 1st leaf |
| IN1 | internode of 1st leaf |
| LS1 | sheath of 1st leaf |
| LL1 | ligule of 1st leaf |
| L1 | blade of 1st leaf |

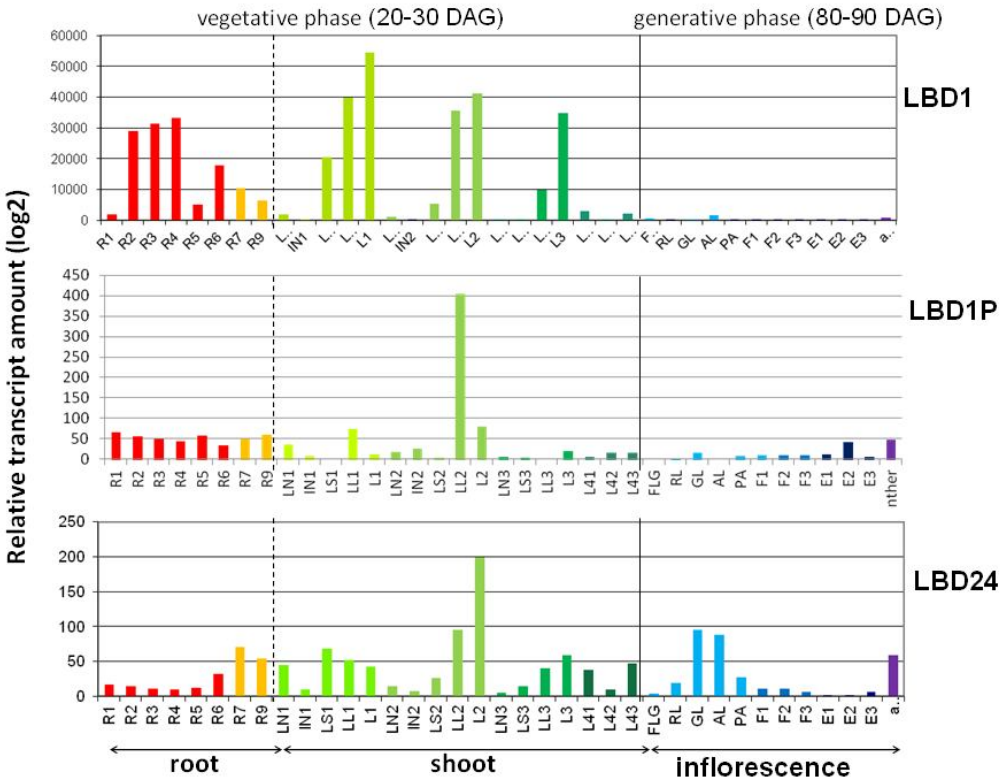
| | |
|---|---|
| LN2 | node of 2nd leaf |
| IN2 | internode of 2nd leaf |
| LS2 | seath of 2nd leaf |
| LL2 | ligule of 2nd leaf |
| L2 | blade of 2nd leaf |
| LN3 | node of 3rd leaf |
| LS3 | seath of 3rd leaf |
| LL3 | ligule of 3rd leaf |
| L3 | blade of 3rd leaf |
| L41 | basal segment of 4th leaf balde |
| L42 | midle segment of 4th leaf blade |
| L43 | apical segmant of 4th leaf blade |

| | generative organs |
|---|---|
| FLG | blade of flag leaf |
| RL | rachilla |
| PA | palea |
| AW | awn of palea |
| GL | glume (lemma) |
| F1 | 1st-2nd flower of spikelet |
| F2 | 3rd-4th flower of spikelet |
| F3 | 5th-6th flower of spikelet |
| S1 | 1st-2nd seed of spikelet |
| S2 | 3rd-4th seed of spikelet |
| S3 | 5th-6th seed of spikelet |
| E4 | anther |

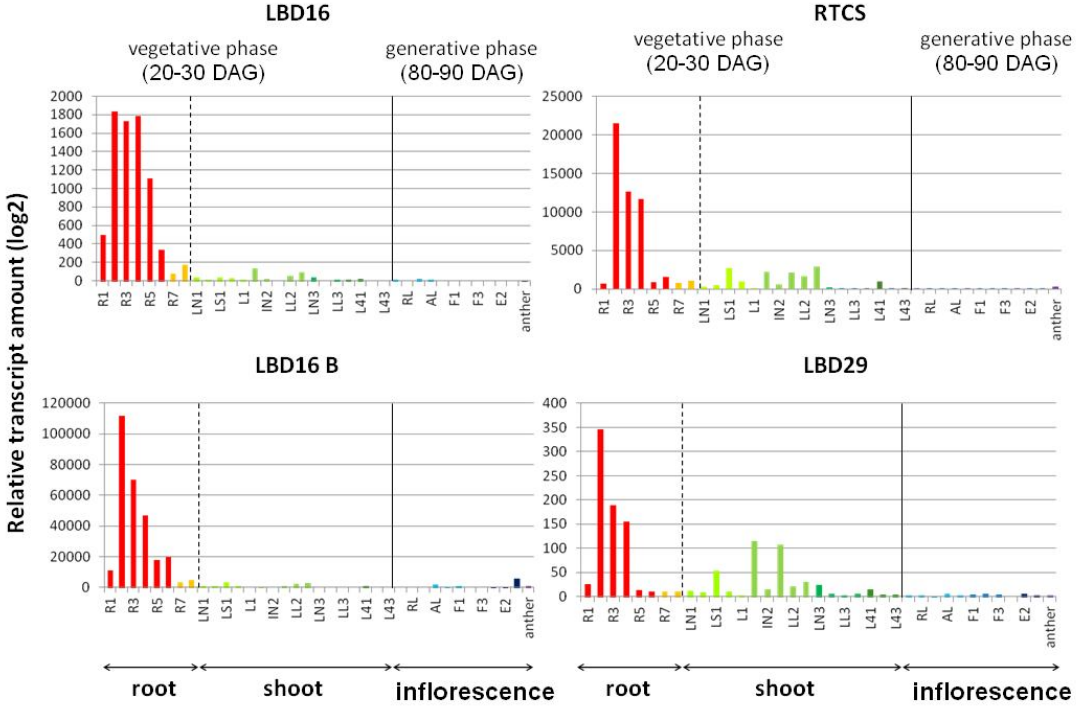
The transcript profiles of the studied genes:



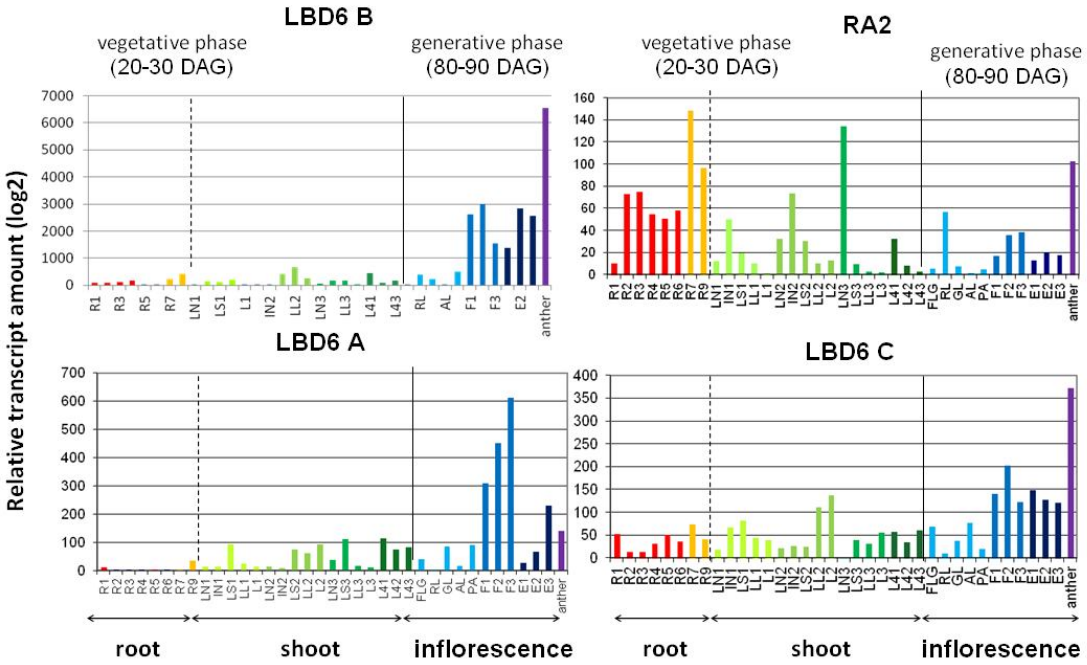
# Expression pattern of LBDs in subclass D



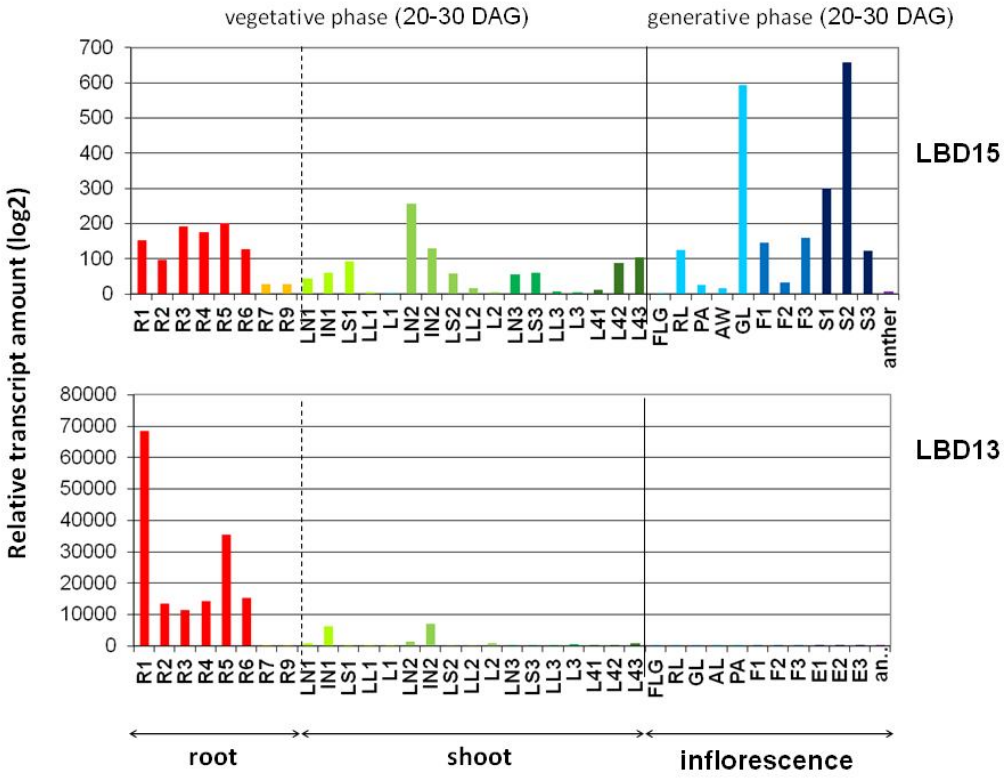

# Expression pattern of LBDs in subclass B



# Expression pattern of LBDs in subclass E




# Expression pattern of LBDs in subclass C



To date, sixteen genes coding for LOB domain transcription factors were characterized in details at transcript level. Various organ specificities were found, one of them possesses exceptionally high root tip specificity. We detected remarkable, drastic transcriptional regulation on the expression of these genes: several hundred fold differences among samples were very common; even 70 thousand fold inductions above background level was detected in the case of Bd2g34520 (LOB13).

During our previous research on *Medicago truncatula*, yeast two-hybrid interaction screen revealed a cDNA which codes for a LOB-domain transcription factor that interacts with cyclin A. This finding gave us the working hypothesis that the interaction between LOB-domain proteins and CDK-cyclin complexes may be an important link of differentiation and cell cycle regulation.

Therefore two putative orthologues (LOB13 and LOB15) of the previously found *Medicago* gene were selected from the 24 member gene family for the most detailed further studies.

The LOB13 is mainly expressed in root parts especially strongly expressed in root tip. The Bd2g53690 (LOB15) is more pronounced in developing seeds and awns but also expressed moderately in roots and in internode above the second leaf. These results confirm the findings in Arabidopsis and go far beyond that because of the much higher details.

Beside the LOB-domain coding genes, the expression profile of an A-type cyclin (Bd4g06827.2) was also determined. According to its putative function (initiation of cell division) and the complementary expression pattern to LOB15 (high expression in young leaves and in glumes where LOB15 exhibit very low expression) it may be a negative regulator of LOB15.

Yeast two-hybrid interaction tests confirmed that LOB13 of *Brachypodium* also interacts with a cyclin-A, so the link seems to be present not only in dicots but monocots as well. Capability to dimerize or to interact with other members of the LOB-domain family was also found.

**Transformation system in *Brachypodium***

For in depth studies on gene regulation and determination of gene function as well as reverse genetic studies, it was essential to establish genetic transformation system of *Brachypodium.* We initiated the adaptation of two type of *Agrocterium*-mediated transformation methods: one is based on co-cultivation of embryo derived calli; while the other uses direct infection of embryos via wounding. We could progress with both methods, but the first one using embryogenic calli turned to be more efficient in our hand.

The Bd21 line is used now for transformation. Immature embryos are isolated and put on callus forming medium. Normally, approximately 90% of the embryos produce usable callus tissue. Smaller embryos (around 0,3 mm diameter) develop higher quality calli. Two round of multiplication can be done in order to increase the starting material for transformation. Calli are chopped into small pieces. It improves the transformation efficiency and does not decrease the regeneration capability (as was proved by the regeneration of control calli). The regeneration of embryos after co-cultivation was successful with most of the media and hormone combinations and the further *in vitro* and greenhouse cultivation of the regenerated plantlets finally resulted in healthy and fertile plants. Details of the improved and optimized protocol can be found in **Appendix 2.**

Based on the above method, genetically modified *Brachypodium* lines were produced. Using specific oligonucleotide primers, both the promoter regions from genomic DNA and the coding sequences from cDNA were PCR amplified. The obtained PCR fragments were subcloned and sequenced to verify them. Plant transformation constructs were made both with LOB13 and LOB15 genes using binary vector system for Agrobacterium mediated transformation:

- Promoter::GFP reporter gene fusions
- UBI promoter:: LOB-ox (overexpression) coding sequence fusions
- UBI promoter:: LOB-si (silencing) coding sequence fusions

Transformations were successfully carried out with LOB13 promoter::GFP reporter constructs; numerous independent transformed lines were regenerated. Fluorescent microscopy analysis of the transgenic lines confirmed that LOB13 gene is expressed in

the root tip as we determined in the transcript profile using quantitative RT-PCR and also revealed promoter activity of this gene in stamen. Preliminary analysis of transgenic plants carrying UBI promoter:: LOB15-ox construct shows retarded plant development, various morphological abnormalities indicating that the ectopic expression of LOB15 successfully interfered with plant development.

Beyond the publications published already in the frame of this grant (five of them peer-reviewed), three manuscripts are still to be published in peer-reviewed journals based on the work carried out in the frame of the current project:

**Szécsényi et al.** Natural variation in root architecture of *Brachypodium distachyon* accessions during moderate water-deficit stress. **Submitted to Planta, see Appendix I**

**Zombori et. al.** Development and optimization of an efficient *Agrobacterium*-mediated transformation method in *Brachypodium distachyon.* **Ready for submission, see Appendix II**

**Gombos et. al.** Detailed transcript profile of all the members of the LOB-domain transcriptional factor gene family in *Brachypodium distachyon*. **In preparation.**

**Future plans:**

The basic research questions for our future work are the followings:

We aim to unshade functional roles of the different LOB types with special emphasis on the ones influencing root architecture. How lateral organ boundary determining transcription factors can have effect on the mitotic activity of the meristematic cells? *Brachypodium* research is to be extended towards the relation of root development and cell cycle. Therefore, we aim to reveal the cell cycle gene families (CDK, cyclin, E2F/DP, retinoblastoma) of *Brachypodium.* Thanks to the advance of genomics on *Brachypodium*, putative miRNA genes are already annotated. We also address the question which miRNA genes may be involved in the transcript level regulation of these cell cycle regulatory genes in roots? A recently started OTKA grant (K109719) supports these directions.

**Appendix:**

**Appendix 1 – manuscript submitted to Planta:**

Mária Szécsényi, Zoltán Zombori, Magdolna Gombos, Györgyi Sándor, János Györgyey

Natural variation in root architecture of *Brachypodium distachyon* accessions during moderate water-deficit stress

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**Main Conclusion**

Nodal roots have pivotal influence on the adaptation of various *Brachypodium* accessions during drought stress, highlighting their central role in the plasticity of root architecture during water limitation.

**Abstract**

Being a *Pooideae* crop plant model, a set of natural accessions of *Brachypodium distachyon* was examined under control and water-limited conditions to reveal their developmental responses in the case of different root types. Two experimental systems were set up with the following goals: (1) to perform a hierarchical cluster analysis on the accessions grown in pots based on the changes in the values of shoot and axile root parameters, and (2) to follow the temporal and spatial development of the selected accessions' root system with and without watering in rhizoboxes. In order to classify accessions based on the most discriminative parameters, principal component analysis was done on data obtained from pot experiments. Regarding the most discriminative traits in the pot experiment, such as the height and weights of shoots together with coleoptile node axile root diameter and leaf node axile root number and total length, six clusters were determined showing various combinations of alterations in the above

mentioned traits. The six accessions selected from each cluster showed changes in different root types, verifying their tolerance to drought stress. Our results support the importance of nodal roots in the differentiation of various *Brachypodium* accessions during drought stress, highlighting their central role in the plasticity of root architecture during water limitation.

**Keywords**

Drought stress, native accessions, PCA, rhizobox, root development

## Introduction

Drought stress is a world-wide agricultural problem, especially in crop production. Improving agricultural productivity by creating drought-tolerant crops to reduce agricultural use of fresh water resources is one of the main goals of plant biologists and crop breeders. However, important cereal crops grown at temperate climate, such as wheat, barley, rye or oat can have some disadvantageous features when either physiological or molecular studies are conducted on them. In the laboratory, their size, both above and below ground can be an obstacle as well as their life-cycle which, e.g. in case of wheat is usually 159-190 days in the field and approximately 50 days less in controlled environment rooms (www.cerealsdb.uk.net). In addition, genome sequences of these cereals are very large. Furthermore, the allohexaploidy of wheat with its huge genome size containing an enormous number of repeated elements together with its complicated transformability can set back the understanding of the genetic background of physiological events. Comparing the structure of the above ground and subterranean parts of the wheat plant, the complexity due to the existence of various root types makes the root architecture analysis that much harder.

When *Brachypodium distachyon* (L.) Beauv. became the new model plant for temperate grasses, a tremendous opportunity presented itself in the analysis of important cereal crops, such as wheat, barley and oat, considering the close genetic relationship between these species and *Brachypodium* (as reviewed by Garvin et al. 2008). The small plant size, the small and fully sequenced genome (272 Mbp), the short life-cycle (up to 3 months), the easy transformability by *Agrobacterium*, the large genetic variability, and the growing number of T-DNA (Bragg et al. 2012) and TILLING mutant populations (http://urgv.evry.inra.fr/UTILLdb) make working with *Brachypodium* more straightforward compared to crops from the *Pooideae* family, which allows us to understand the latter ones better indirectly. Beside the above-mentioned advantages, *Brachypodium* even possesses a simpler root system than wheat, albeit showing high degree of both anatomical and developmental similarity with the wheat root system (Watt et al. 2009). According to the comparison of Bd21 (the *Brachypodium* reference line) and wheat root system, "(1) the same types of axile roots; (2) nearly identical timing of emergence of axile roots; (3) very similar branch root types, sharing five types, with *Brachypodium* having two extra types; and (4) deep roots comprising of mainly branch

roots". Therefore, *Brachypodium* is an excellent model to study cereal root system genetics and function both at seedling level and the mature phase of the plant.

In our study, a group of *Brachypodium* accessions was analyzed that were previously characterized on the basis of shoot parameters under drought stress by Luo et al. (2011) (listed in Table 1) The pot-based experimental system that was developed for the analyses of various wheat genotypes (Szécsényi et al. 2013) was suitable for studying the phenotypic behavior of the plants in terms of relationship between shoot growth and axile root features under control and drought-stressed conditions. Based on the data obtained from the pot experiment, a narrowed fraction of the accessions was selected to be grown in rhizoboxes allowed us to monitor the development of their root system in detail with or without watering. Notice that the two experimental systems differ in (1) the developmental stage of the plants, (2) growth medium and (3) the degree of water limitation. Performing detailed analysis on root system of *Brachypodium* plants can allow us to reveal developmental strategies of grasses during water limitation that can take us closer to understanding tiny but important details about the plasticity of the wheat root system.

## Materials and methods

### *Plant material and growth conditions*

Thirty-one accessions of purple false brome (*Brachypodium distachyon* (L.) P.Beauv.) were provided by David Garvin (USDA-ARS) and the U.S. National Plant Germplasm System (Table 1). These accessions were characterized under control and adverse watering conditions by applying two methods. After a five-day-long stratification period, ten seeds per pot (diameter: 12 cm, volumetric capacity: 1.2 l) were sown at a depth of 1.5 cm into a wet mixture of sand and perlite (Pannon-Perlit Ltd., Hungary) (2:1). Seedlings were singled out 9 days after germination resulting in four plantlets per pot in a cross-like pattern. Plantlets were irrigated with 0.5-fold strength Hoagland solution adjusted to 80% field capacity (FC) for three week after germination. At the three-leaf stage, drought stress was induced by reducing the water supply for two weeks. After reaching the desired 40% FC (half of the control samples) achieved by continuous drying avoiding irrigation, water content was maintained through embedded dripping tubes using distilled water. Meanwhile, control samples (at 80% FC) at the three-leaf stage were watered using 1-fold Hoagland solution till stressed plants reached 40% FC followed by irrigation at surface with distilled water until the end of the experiment. Plants were grown in growth chamber at 19 ºC:23 ºC (night:day) under a cycle of 18 h illumination (140 µmol $m^{-2}$ $s^{-1}$) and 6 h darkness. The relative humidity of the air was set to 50–60%. Samples of 31 *Brachypodium* accessions were harvested for biplot analysis.

Root architecture was monitored on plants grown individually in rhizoboxes (29x20.5x1.3 cm) made of a transparent glass and a plastic sheet filled with vermiculite (Pull Rhenen, The Netherlands) and perlite (Pannon-Perlit Ltd., Hungary) in a 1:1 ratio. In order to keep roots growing next to the glass surface, roots were separated from the

growth medium by using a nylon net (55 μm, Sefar AG, Heiden, Switzerland) that is impermeable for roots but allows root hairs to reach the medium. The rhizoboxes were stored at an angle of 45°. Nine plants for each accession×treatment (A×T) combination were grown under the same growth conditions as plants grown in pots, except that a group of plants were either irrigated daily (control) or were left to dry (stressed). The experiments lasted approximately for three weeks after sowing; until the roots of two or more plants of the set of nine reached the bottom of the rhizobox.

*Parameters measured on plants grown in pots*

In pot-based experiment, both shoot and root parameters were recorded. After 2-week-long drought stress, the fresh weight (FW) of the green part and shoot height (SH) were recorded. For determining the dry weight (DW) of the shoots, they were dried in an oven at 80°C for 24 h. In case of roots, the length and diameter of a 1 cm long root tip were measured in primary axile root (PR), coleptile node axile root (CNR) and leaf node axile root (LNR). The diameter (d) was calculated from pixel numbers using digital photos taken by stereo microscopy (1600x1200 pixel resolution, 1x optical zoom, 90x zoom then 1 pixel=0.88 μm). Root numbers (No.) were determined in the case of CNRs and LNRs.

*Parameters measured on plants grown in rhizoboxes*

In rhizoboxes, the growth rate (GR) of the roots was monitored by marking the position of the root tip on the glass plate at the same time point of every day. In the case of PR, observation started on the third day after sowing. After three weeks, the length of PR (PR-L), average and total length of CNRs (CNR-Lsum), LRs (LR-L and LR-Lsum, respectively) and LNRs (LNR-Lsum) was either measured or calculated. The number (No.) and exact time of emergence (TOE) of first order LRs (LR-No., LR-TOE), CNRs (CNR-No., CNR-TOE) and LNRs (LNR-NO., LNR-TOE) was recorded. Total root length (TRL) was determined as the summed length of all the root types. Using the image analyzing software ImageJ 1.47v (Abramoff et al. 2004), gravitropic set-point angle of lateral roots (GSA), angle of displacement of the PR's tip (degree of skewing - angle B), PR's straightness (S-PR) as well as the area (CHA), height (CHH) and width (CHW) of the convex hull enclosing the root system were determined. Convex hull of a *Brachypodium* root system together with GSA and angle B are explained in Online Resource 1. S-PR along with vertical growth index (VGI) and horizontal growth index (HGI), an indication of PR skewing, were calculated according to Grabov et al. (2005). For the green part of the plants, height (SH) was measured at the end of the experiment. PR:S-L ratio was calculated from the length of PR and shoot. For the reader's convenience, abbreviations of parameters measured either in pot-based experiment or rhizoboxes are listed in Table 2.

*Statistical data analyses*

In order to select those parameters which show genetic variation or were influenced by drought stress treatment or even have a significant A×T interaction two-way ANOVA with replications was carried out using MS Excel. Determining the most discriminative traits among the accessions examined, the selected parameters were further analyzed by principal component analysis (PCA) using PAST v.2.17c statistical software (Hammer et al. 2001). Accessions were classified by hierarchical cluster analysis applying the software PermutMatrix 1.9.3 (Caraux and Pinloche 2005). Pearson's correlation coefficients were determined by using MS Excel followed by a two-tailed probability calculation (Soper 2014).

## Results

*Significant alterations upon drought stress and differences between 31 accessions in the pot experiment*

Among the traits measured in the pot experiment, shoot height together with LNR number was significantly affected by water limitation, which also varied between accessions and a significant A×T interaction was observed, as well (Table 3). Other shoot and root parameters showing either drought stress or genotypic "control" without a significant A×T interaction were also considered. Hence, shoot weights, PR:S-Lratio, PR-d and CNR-d, as well as LNR-Lsum were influenced by both limited irrigation and genotypic diversity, while CNR-Lsum and CNR-No. showed a discrepancy among the accessions without any drought affection.

*LNRs and CNRs are the most discriminative root components among the accessions in the pot experiment*

In order to group different *Brachypodium* lines according to their response to water deficit, PCA was performed using traits listed in Table 3 on the plants that were watered with and without restriction (Fig. 1). The first two components contained the majority (82%) of the total variance: PC1 was related to SH, FW and DW as well as LNR-No. while PC2 was mainly driven by LNR-Lsum together with CNR-d (Online Resource 2).

In order to represent the changes caused by water limitation in a given accession, we determined the length and slope of the vectors linking control and treated samples of an accession, reflecting the degree and direction of the changes in the discriminative parameters (Online Resource 3). These vector features were the bases of the classification of the accessions using hierarchical cluster analysis, resulting in six distinguishable groups (Fig. 2). An overview of the clustering shows that all of the accessions moved

towards smaller values along the PC1 axis as a direct effect of the water limitation (Online Resource 3). Hence, the main differences between the clusters were (1) the degree of alterations along the PC1 axis together with (2) changes if there were any along the PC2 axis. Cluster 4 accessions, such as Adi-9, Bd2-3, Bd21, BdTR3H, Gaz-2, Gaz-8, Koz-4, and Tek-2, moved, practically, in parallel with the PC1 axis which corresponds to no changes along the PC2 axis. A common feature of accessions in cluster 1 (Adi-10, BdTR9B, BdTR13E, Gaz-1, Gaz-6, and Koz-1) and cluster 6 (Adi-14, Bd21-3, Bd29-1, BdTR2A, BdTR2B, Gaz-5, and Kah-6) was the decrease along the PC2 axis coupled with either a greater decrease along PC1 (cluster 6) or a more remarkable decline along PC2 (cluster 1). Otherwise, a similar increase along PC2 was observed in the case of cluster 2 (Bd18-1, Bd30-1, BdTR2C, and Kah-2), 3 (Adi-4, Bd3-1, and Kah-1), and 5 (Bd1-1 and Kah-3) but in parallel with an increasing drop along the PC1 axis, respectively.

Hence, cluster 1 plants were remarkable in that they showed either a decrease or no change at all, rather than an increase in CNR-d together with relatively high values of LNR traits during water withdrawal compared to the plants of other clusters. On the other hand, members of cluster 2 showed propensity in increasing their CNR-d and decreasing the FW and SH but not their DW. The “specialty” of members of cluster 3 was a decrease in the value of LNR parameters and, like in cluster 2 plants, DW also did not change under stressed conditions. Similarly to the previous cluster, accessions belonging to cluster 4 also produced much less LNRs during water limitation and a significant decline in the value of shoot parameters in most of the accessions during drought stress was observed. Members of cluster 5 had less LNRs during drought stress and the latter accession had the highest increase in CNR-d, but Bd1-1 had a tendency only to increase its diameter of this root type. Similar to cluster 4, most of the accessions of cluster 6 displayed a significant decrease in shoot parameters in most of the accessions during water limitation compared to the other clusters. Regarding root parameters of this cluster, CNR thickness, similarly to plants of cluster 1, showed rather a tendency of reduction or was not affected by drought stress while mostly decreases in LNR traits were observed. Comparing BdTR8I as the lonely member of the hierarchical clustering to the six clusters, stressed plants had no LNRs compared to the control ones which had more than any of the other accessions. In addition, stressed BdTR8I plants had less green tissues along with no change in CNR-d related to the control ones. Nevertheless, each cluster also had at least one accession which had no LNRs during water withdrawal except cluster 2.

*The relationship between various shoot and root traits under control and water-limited circumstances*

In the pot experiment, based on the correlation analysis of the six clusters presented in Figure 2, significant relationships between the traits that discriminate the accessions were recorded [summarized in Online Resource 4 (sheet 1)]. Due to the low number of accessions, cluster 3 (n=3) and 5 (n=2) did not show any significant correlations in the six parameters. Hence, the other four clusters are described in this section. Putting the values together into a single table (Table 4), SH positively correlates

with either the FW or DW of green parts in two of the clusters: control samples of cluster 6 and drought-stressed samples of cluster 4. In addition, while all the samples of cluster 4 and 6 showed positive correlation between FW and DW, only the control samples belonging to cluster 1 and 2 showed this correlation. Moreover, FW also showed a positive correlation with LNR-No. in control plants of cluster 4 ($r^2$=0.80, $P$=0.017) and with CNR-d in control plants from cluster 6 ($r^2$=0.88, $P$=0.009). On the other hand, DW of the above-ground part was positively correlated with different traits of LNRs. It showed a correlation of 0.72 ($P$=0.044) with LNR-No. in control plants of cluster 4, and a correlation of 0.77 ($P$=0.043) with LNR-Lsum in cluster 6. Regarding the relationship between the two LNR parameters, the number and total length of this root type, they show a positive correlation with plants in both types of samples from three clusters (1, 4, and 6) but not with plants of cluster 2. However, the low number (n=4) of accessions is the probable reason of the almost positive correlation in control plants of the latter group of accessions but not in stressed plants.

*Rhizobox – root architecture of six accessions representing the clusters*

In order to examine the root architecture of the accessions classified into various clusters based on data obtained in the pot experiment, six accessions, namely Gaz-6 (cluster 1), Bd18-1 (cluster 2), Bd3-1 (cluster 3), Bd21 (cluster 4), Bd1-1 (cluster 5), and Bd21-3 (cluster 6) were selected to grow in a rhizobox with and without watering. The selection was primarily based on sequence data available at www.brachybase.org. The genome of Bd21 as the reference line along with Bd1-1, Bd3-1 and Bd21-3 are fully sequenced (Gordon et al. 2014), and also EST sequences are available from the root tissue of Bd18-1, Bd3-1, Bd21, and Bd21-3. Furthermore, TILLING mutant population was created from Bd21-3 (http://urgv.evry.inra.fr/UTILLdb). So far, none of the accessions from cluster 1 was involved into genome sequencing. Therefore, Gaz-6 was chosen based on the length of PR being the longest one among the accessions grown in the pot-experiment during water limitation. Similar to the drought treatment applied in the pot experiment, most of the root traits were affected by the treatment compared to the control samples with occasional simultaneous genotypic variability (Table 5). A×T interaction was observed in PR-L, PR-GR, CNR-No., LNR-No., LNR-Lsum, GSA, and TRL. In contrast with the pot experiment, SH showed significant genotypic variation but no drought dependence.

*CNR and LR traits along with TRL and convex hull parameters as main discriminators of the six accessions in the rhizobox*

Similarly to pot experiment, all the traits that showed either significant genotypic variation or alterations without irrigation (Table 5) were involved in PCA on the plants grown under control and water limited conditions (Fig. 3). The first two components contain 85% of the total variance: PC1 was strongly related to CNR-No., CNR-Lsum, CNR-TOE as well as TRL while PC2 was mainly driven from LR-L together with CHA

and CHW (Online Resource 5). Gaz-6 (cluster 1) together with Bd21 (cluster 4) showed similar responses regarding the root traits presented in PCA, namely that a tendency of decrease in CNR-No. and CNR-Lsum together with a significantly less TRL was measured in both accessions. However, the size of the convex hull decreased only in stressed Bd21 plants. Furthermore, significantly less CNRs were observed in two accessions, such as Bd1-1 (cluster 5) and Bd18-1 (cluster 2). In the case of the latter one, no CNRs were developed at all without watering. In these two accessions only the decrease in numbers was monitored in TRL. On the other hand, no change was detected either in CNR parameters or in TRL in the case of Bd3-1 (cluster 3) and Bd21-3 (cluster 6). LR-L is the key point in the comparison of the latter two accessions: it was significantly decreased in Bd3-1 but was not affected in Bd21-3 by water-limited conditions. Similarly to Bd3-1, significantly shorter LRs were developed in stressed Bd1-1 plants as well. Roots of Bd18-1 plants covered the biggest area of the soil among the six accessions being examined even with a significant decrease of CHW during water-limited conditions as well as with a significant increase of CHA in Bd21-3 plants.

*Correlations between root traits of the six accessions grown with and without watering*

Online Resource 4 (sheet 2) gives details about the correlations among the distinct root parameters between the six accessions belonging to a certain cluster. Regarding the five most discriminative traits as a product of PCA, CNR parameters along with TRL had significant correlations but not LR-L (Table 6). Both watered and non-watered samples showed a positive correlation between CNR-No. and CNR-Lsum [$r^2$=0.85 ($P$=0.032) and $r^2$=0.91 ($P$=0.012), respectively]. However, the correlation between CNR-TOE was negative with both the number and total length of this root type only in non-watered plants. These three CNR traits correlate to TRL on a similar manner (CNR-TOE negative, CNR-No. and CNR-Lsum positive), in stressed plants only.

*Significant changes in values of root traits in the six representative accessions*

Besides the root parameters that were capable of significantly discriminating the six accessions, significant alterations in root traits as an effect of water limitation was distinct in the genotypes examined in rhizoboxes (Table 7). Regarding first the discriminative CNR parameters, significant changes were detected in these parameters upon water withdrawal compared to the control plants in two accessions: Bd1-1 from cluster 5 and Bd18-1 from cluster 2. While a more or less slight decrease in CNR-No. (30%), CNR-GR (22%) and CNR-Lsum (47%) was observed in Bd1-1, no CNRs were developed at all in Bd18-1. On the other hand, LR-L decreased due to water limitation in Bd1-1 from cluster 5 (28%) and Bd3-1 from cluster 3 (45%). However, the number of LRs increased (+125%) in the latter genotype. Nevertheless, the other accessions (Gaz-6 from cluster 1 and Bd21 from cluster 4) also showed significant alterations in traits of LRs of plants grown under water-limited conditions compared to well-watered ones. In contrast to Bd3-1, the LR-No. of these two genotypes decreased in Gaz-6 into the half of

the controls' and in Bd21 by 36% together with a reduced LR-Lsum (-36% and -54%, respectively). Obviously, based on a strong correlation between LR-No. and LRD [Online Resource 4 (sheet 2)]), similar changes were detected in LRD of the three genotypes. Moreover, LR-TOE increased only in Gaz-6 (28%). TRL, as another discriminative parameter of the six accessions, was decreased by water deficit in Gaz-6 and Bd21 to a similar degree.

Besides these changes, the other two root types were also affected in plants grown during water-limited conditions. PR-GR as well as PR-L increased in Bd1-1 (17% and 14%, respectively) and Bd21-3 from cluster 6 (28% and 21%, respectively), resulting in a higher PR:S-L ratio. In the case of LNRs, hampered growth was noticed in Bd21 and Bd21-3 while in Bd3-1 this effect was the opposite. Notably, no LNRs were detected either in control or stressed plants in Bd1-1 and Bd18-1. In the spatial geometry of the root architecture, a significant increase in CHH was observed in Gaz-6 (15%) and Bd21-3 (23%), while CHW was similarly reduced in Bd18-1 and Bd21 (23 and 24%, respectively). In the latter genotype, the decreased CHW resulted in a shrinkage of one-third of CHA covered by the root system. On the other hand, in Bd18-1, the narrower (-23%) area occupied by the roots was associated with lower HGI (-57%, due to the lower growth angle) and higher VGI (+18%, due to diminished curvature) compared to the control plants. S-PR increased slightly in Bd21. In two accessions, Bd3-1 and Bd21-3, GSA was significantly higher (18% and 14%, respectively) in stressed plants compared to the well-watered ones.

*Relationship of the altered traits with other parameters*

In order to see what other parameters might be changed upon water limitation due to their relationships with the altered traits, correlation coefficients were determined to measure these relationships [Online Resource 4 (sheet 3)]. As a member of cluster 1, the accession Gaz-6 mainly showed LR and PR-related changes upon water deprivation. The declined TRL of Gaz-6 plants is positively associated with the late emerged and shorter LRs under water-limited conditions. On the other hand, increased CHH corresponds to an elevated PR-L as an effect of water limitation after a positive correlation ($r^2$=0.88, $P$=0.004) was observed for it. It is also confirmed by a negative correlation between the shoot height and PR:S-L. In parallel with a deeper convex hull, a straighter PR can be assumed based on the positive correlation between CHH and VGI ($r^2$=0.80, $P$=0.017). A straighter PR was observed in Bd18-1 from cluster 2, as well, that presumes a longer PR, therefore a deeper area covered by the root system (CHH) based on their positive correlations with VGI upon water limitation [$r^2$=0.67 ($P$=0.048) and $r^2$=0.81 ($P$=0.008), respectively]. Furthermore, Bd18-1 plants with straighter PR presumably have faster elongating LRs under water-limited conditions. The decreased CHW (narrower area) presumes less LRs in total length ($r^2$=0.77, $P$=0.015) emerged at a larger angle ($r^2$=-0.70, $P$=0.036) along with a shorter TRL ($r^2$=0.76, $P$=0.017). Changes were detected mostly in LNR and LR traits in Bd3-1 belonging to cluster 3. Interestingly, the increased number and length of LNRs correlates positively with SH [$r^2$=0.82 ($P$=0.007) and $r^2$=0.70 ($P$=0.036), respectively]. However, no increase in this parameter could be demonstrated

by this relationship. On the other hand, more LRs under drying circumstances correlate to faster elongation ($r^2$=0.79, $P$=0.011) and longer PR ($r^2$=0.78, $P$=0.013), which is opposite to the average length of LRs [$r^2$=-0.79 ($P$=0.011) and $r^2$=-0.76 ($P$=0.017), respectively]. However, in contrast with SH, there is an increase in both PR parameters, although it is not significant (Online Resource 6). Furthermore, the elevated number of LRs together with higher LRD assumes a longer TRL [$r^2$=0.67 ($P$=0.048) and 0.70 ($P$=0.036), respectively]. Bd21 as a member of cluster 4 shows decreased values of LNR and LR parameters. The former one, such as less LNRs, presumes slower elongating PR ($r^2$=0.68, $P$=0.044). It has also been confirmed by other positive correlations, either with growth rate or length of PR, such as TRL and CHA being decreased by water deprivation. Moreover, slightly elevated S-PR presumably correlates with a more pronounced gravitropism [angle B: $r^2$=-0.86 ($P$=0.003) and HGI: $r^2$=-0.83 ($P$=0.006)]. Similar correlation of S-PR was found with a narrower area covered by the root system (CHW; $r^2$=-0.69, $P$=0.040). Bd1-1, the representative accession of cluster 5, shows alterations in PR, CNR and LR traits. The more rapidly elongating and longer PR is associated with shorter CNRs ($r^2$=-0.70, $P$=0.036). Furthermore it suggests, based on significant correlations, more slowly elongating LRs ($r^2$=-0.73, $P$=0.026) and that the PR is less skewed and straighter. These relationships also include a longer but narrower root system ($r^2$=-0.74, $P$=0.023)). The decreased number of CNRs also presumes a less skewed PR [angle B: $r^2$=0.77 ($P$=0.015), HGI: 0.78 ($P$=0.013)]. Altogether, the increased growth of PR hampers the development of CNRs. Bd21-3, a member of cluster 6, displays predominant changes in PR and LNR traits. Ambiguously, decreasing number of LNRs has a correlation with the growth rate of PR ($r^2$=0.72, $P$=0.029) as well as skewing parameters [angle B: $r^2$=-0.69 ($P$=0.040) and HGI: -0.75 ($P$=0.020)] assuming a slower elongation and more skewed PR under water-limited conditions. However, the opposite alterations can also be observed: a significantly faster PR that is less but not significantly beveled. The latter fact is confirmed by a negative correlation between the increased CHH and either angle B ($r^2$=-0.89, $P$=0.001) or HGI (-0.86, $P$=0.003). Nevertheless, a deeper root system based on a higher CHH presumes straighter PR, as well. On the other hand, increased GSA as the larger growth angle of LRs presumably results in a smaller area covered by the root system of Bd21-3 that is the opposite of the growing CHA albeit in a non-significant way (Online Resource 6).

## Discussion

An efficient uptake of soil water is essential for plant growth in water-limited periods of plant life. Plasticity of the root system facilitates exploring water lying deeper in the soil or dispersedly in patches. While the root system architecture (RSA) is primarily based on the primary root growth and lateral root formation in case of the dicot plant model *Arabidopsis*, in temperate grass model *Brachypodium*, RSA is characterized more by curving, elongating, and branching (Lynch 1995).

In our study, two approaches were used to investigate the plasticity of roots of various *Brachypodium* accessions. The pot experiment was important in the classification of the accessions into clusters based on the changes in values of traits of the axile root

types. However, neither temporal nor spatial development of the roots could be tracked reliably in the pots. Therefore, a rhizobox experiment was set up, conforming to the root architecture plasticity of a single plant of a sample accession from each cluster, determined on the basis of changes in growth patterns in pots, under water-limited conditions compared to the well-watered ones. However, due to the differences between the two experimental setups, the two approaches cover different (1) developmental stages, (2) stress fashion, and (3) growth medium. Hence, data obtained from rhizoboxes complement data obtained from pot experiments instead of repeating the latter's result as a part of a more sophisticated system.

*Signs of the effect of drought in the above-ground parameters*

Plants grown under water-limited conditions in pot experiments showed reduced shoot growth compared to control plants. This well-known, general response of plants to diminished quantities of water can be the result either drying of the surface (Blum et al. 1991) or the soil strength resulting from soil drying (Whalley et al. 2006). In pots, both effects could lead to loss in green biomass. In rhizoboxes, a significant decline was measured only in the SH of Bd21 plants, highlighting the different strength of water-deficit stress in the two experimental systems being used. Considering that vermiculite remains loose when dries, only the surface drying can be the reason for the decreased plant height of Bd21. How do we know then that plants grown in rhizoboxes had been affected by water limitation? First of all, it was seen from the rearrangement of their root architecture, resulting in greater elongation of the primary root (Bd1-1, Bd21-3), as well as a smaller number of shorter lateral roots (Gaz-6, Bd21 and Bd3-1, Bd1-1, respectively) or a less curved and more gravitropic primary root (Bd18-1). Hence, our rhizoboxes are suitable tools for analyzing early steps in drought avoidance by different *Brachypodium* accessions.

*Different root types*

In both experimental setups, CNR traits turned out to be pronounced features among the accessions under examination. While the root diameter distinguishes plants grown in pots, the number, and total length along with the time of emergence are the discriminative features of this root type comparing the six accessions grown in rhizoboxes. The diameter of a root, especially the apical diameter, can be associated with, for instance, axial growth (e.g. Hackett 1973), water transport properties (e.g. Varney and Canny 1993), and penetration ability (e.g. Misra et al. 1986). Diameter showed a positive correlation with the size of root cap and columella as well as with growth direction in rice nodal roots (Abe and Morita 1994). Some studies highlight the strong link between the diameter and penetration ability, describing the increased diameter as a response to mechanical impedance caused by the increased soil strength of drying soil due to the capillary forces (Bengough et al. 2011). In our study, sand made up two-thirds of the soil used in pots, which can result in the increased mechanical strength of the soil, as

described by Whalley et al. (2006). Hence, accessions possessing significantly increased CNR-d, such as Kah-3 from clusters 5, probably reached a compacted layer in the soil caused by drying that was too strong for it which is supported by its decreased length of CNRs (Hodge et al. 2009). A similar but not significant effect of soil strength could be observed in Bd1-1. On the other hand, some of the accessions belonged to clusters 1 and 6 showed a tendency of decrease in their CNR-d under water-limited conditions. Decreased root diameter has been proposed as a trait for increased water acquisition of the plant under drought stress (Wasson et al. 2012). Furthermore, based on the study of Luo et al. (2011), three accessions, namely Bd21-3, BdTR13E and Gaz-5 were described as moderately tolerant, whereas the others (Gaz-1, Gaz-6, Kah-6, and Koz-1) as susceptible based on their above-ground responses subjected to drought stress. We may hypothesize that hydrotropism, defined as moving toward water in drying soil is only one part of drought avoidance but taking up water through aquaporins can limit this strategy (Di Pietro et al. 2013). Nevertheless, at this point we could find a linkage between the two experiments, despite experimental differences; namely that Gaz-6 and Bd21-3 (members of cluster 1 and 6, respectively) tend to avoid drought stress with increased root elongation. In rhizoboxes, a deeper PR was detected in both accessions together with Bd1-1 without watering that is one of the proposed key elements in crop improvement under water-limited conditions (Wasson et al. 2012; Lynch 2013).

Examining further the idealistic root system of maize adapted to water-limited environments, few and long LRs distributed evenly along the depth axis are preferable, considering their small influence on the carbon budget as well as exploration capability of a larger soil volume (Lynch 2013). Hence, the development of more and shorter LRs, as are developed by Bd3-1 plants under water limitation, looks like an inefficient drought avoidance strategy. In addition, this is also confirmed by the *dig3* (drought inhibition of lateral root growth) *Arabidopsis* mutant that is much more sensitive to drought stress than the wild type (Xiong et al. 2006). Therefore, our results coincide with data of Luo et al. (2011), showing that Bd3-1 is a drought-sensitive accession. Bd18-1 was the other accession examined in rhizobox which was described as the most susceptible to drought, as well, by Luo et al. (2011). However, the root system of Bd18-1 covered the largest area of the growth medium in rhizobox, which can however be a disadvantage under water limited circumstances. Considering the observation published by Campbell et al. (1991), it is suggested that species with a large root area are supposed to be less able to place roots selectively in high-nutrient patches, which could be a trade-off between the ability to explore large soil volumes and that to exploit nutrients or in our case water in depth.

*Concluding remarks*

Shoot length and weight are well-known parameters which demonstrate the detrimental effects of drought stress. Besides these, the principal component analysis revealed that the number and length of the LNRs and the diameter of the CNRs are the appropriate parameters to characterize the response of the root system of *Pooideae* plants during water limitation in the vegetative phase. Based on the cluster analysis on the data

obtained from pot experiment, the six accessions that were selected for rhizobox analysis were suitable for examining different adaptation strategies to water-deficit stress. Therefore, these *Brachypodium* genotypes can serve as good candidates to investigate the genetic and gene expression background of the early response of the root system of *Pooideae* crops grown during water-limited conditions.

## Author Contribution Statement

MS and JG conceived and designed research. MS, ZZ, MG and GS conducted experiments. MS, ZZ and JG analyzed data. MS and JG wrote the manuscript. All authors read and approved the manuscript.

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**Table 1** Description of the *Brachypodium* accessions involved in pot experiment indicating genotypes also grown in rhizoboxes in bold text

| ID | Accession | Location | Growth habit | Drought tolerance based on Luo et al. (2011) |
|---|---|---|---|---|
| 1 | Adi-4 | Turkey (Adiyaman) | Winter | Moderately tolerant |
| 2 | Adi-9 | Turkey (Adiyaman) | Winter | Moderately tolerant |
| 3 | Adi-10 | Turkey (Adiyaman) | Winter | Susceptible |
| 4 | Adi-14 | Turkey (Adiyaman) | Winter | Susceptible |
| **5** | **Bd1-1** | Turkey | Winter | Tolerant |
| 6 | Bd2-3 | Iraq | Spring | Most susceptible |
| **7** | **Bd3-1** | Iraq | Spring | Most susceptible |
| **8** | **Bd18-1** | Turkey | Winter | Most susceptible |
| **9** | **Bd21** | Iraq | Spring | Susceptible |
| **10** | **Bd21-3** | Iraq | Spring | Moderately tolerant |
| 11 | Bd29-1 | Ukraine | Winter | No data available |
| 12 | Bd30-1 | Spain | Spring | Susceptible |
| 13 | BdTR2A | Turkey | Winter | No data available |
| 14 | BdTR2B | Turkey | Winter | Most susceptible |
| 15 | BdTR2C | Turkey | Winter | Most susceptible |
| 16 | BdTR3H | Turkey | Winter | Susceptible |
| 17 | BdTR8I | Turkey | Winter | Moderately tolerant |
| 18 | BdTR9B | Turkey | Winter | Most susceptible |
| 19 | BdTR13E | Turkey | Winter | Moderately tolerant |
| 20 | Gaz-1 | Turkey (Gaziantep) | Winter | Susceptible |
| 21 | Gaz-2 | Turkey (Gaziantep) | Winter | Susceptible |
| 22 | Gaz-5 | Turkey (Gaziantep) | Winter | Moderately tolerant |
| **23** | **Gaz-6** | Turkey (Gaziantep) | Winter | Susceptible |
| 24 | Gaz-8 | Turkey (Gaziantep) | Winter | Susceptible |
| 25 | Kah-1 | Turkey (Kahta) | Winter | Susceptible |
| 26 | Kah-2 | Turkey (Kahta) | Winter | Susceptible |
| 27 | Kah-3 | Turkey (Kahta) | Winter | Susceptible |
| 28 | Kah-6 | Turkey (Kahta) | Winter | Susceptible |
| 29 | Koz-1 | Turkey (Kozluk) | Winter | Susceptible |
| 30 | Koz-4 | Turkey (Kozluk) | Winter | Susceptible |
| 31 | Tek-2 | Turkey (Tekirdag) | Winter | Moderately tolerant |

**Table 2** Abbreviations used in this study

| | |
|---|---|
| SH | Shoot height (mm) |
| FW | Fresh weight of the shoot (mg) |
| DW | Dry weight of the shoot (mg) |
| PR | Primary root |
| CNR | Coleoptile node axile root |
| LNR | First leaf node axile root |
| LR | First order lateral root on PR |
| L | Average length (mm) |
| Lsum | Total length (mm) |
| D | Diameter at the root tip (μm) |
| GR | Growth rate (mm $day^{-1}$) |
| TOE | Time of emergence (the day after sawing when the first root was first viewed) |
| LRD | Lateral root density (number of LRs per PR length, $mm^{-1}$) |
| TRL | Total root length (mm) |
| GSA | Gravitropic set-point angle – angle of LRs to the gravitropic axis (degree) |
| PR:S-L | Ratio of the length of PR and shoot |
| Angle B | Angle of displacement of the PR's tip (degree) |

| | |
|---|---|
| HGI | Horizontal growth index |
| S-PR | Straightness (the length of chord connecting the base and apex of PR normalized onto the length of PR) |
| VGI | Vertical growth index |
| CHA | Convex hull area (pixel number) |
| CHW | Convex hull width (pixel number) |
| CHH | Convex hull height (pixel number) |

**Table 3** Summary of two-way ANOVA of the pot experiment

| | SH | FW | DW | PR:S-L | PR-d | CNR-L | CNR-No. | CNR-d | LNR-No. | LNR-Lsum |
|---|---|---|---|---|---|---|---|---|---|---|
| A | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** |
| T | *** | *** | *** | ** | ** | ns | ns | ** | *** | *** |
| A×T | * | ns | ns | ns | ns | ns | ns | ns | *** | ns |

ns, not significant. * $P$<0.05; ** $P$<0.01; *** $P$<0.001.

**Table 4** Significant correlations between the traits measured in pot experiment

| | SH | FW | DW | LNR-No. |
|---|---|---|---|---|
| FW | 0.78*(6WW) | | | |
| | 0.89**(4D) | | | |
| DW | 0.87*(6WW) | 0.92**(1WW) | | |
| | 0.85**(4D) | 0.97*(2WW) | | |
| | | 0.98***(4WW,D) | | |
| | | 0.94**(6WW) | | |
| | | 0.96***(6D) | | |
| CNR-d | | 0.88**(6WW) | | |
| LNR-No. | | 0.80*(4WW) | 0.72*(4WW) | |
| LNR-Lsum | | | 0.77*(6WW) | 0.98***(1WW,6WW) |
| | | | | 0.91**(4WW) |
| | | | | 0.94**(1D) |
| | | | | 0.99***(4D) |
| | | | | 0.97***(6D) |

Rows and columns associated with SH, CNR-No. and CNR-d, PR:S-L, respectively, were omitted in order to simplify the table. Numbers and letters in parentheses represent the cluster and the sample type (WW, well-watered; D, drought-stressed). * $P$<0.05; ** $P$<0.01; *** $P$<0.001.

**Table 5** Summary of two-way ANOVA - shoot and root parameters from the rhizobox experiment are presented

| Traits | Accession (A) | Treatment (T) | A×T |
|---|---|---|---|
| SH | *** | ns | ns |
| PR-L | ** | * | * |
| PR-GR | *** | * | ** |
| CNR-No. | *** | * | ** |
| CNR-GR | * | ** | ns |
| CNR-Lsum | *** | *** | ns |
| CNR-TOE | *** | *** | ns |

| | | | |
|---|---|---|---|
| LNR-No. | *** | *** | *** |
| LNR-Lsum | *** | ** | *** |
| LR-No. | *** | ns | ns |
| LR-GR | *** | ns | ns |
| LR-L | *** | ** | ns |
| LR-TOE | *** | ns | ns |
| LRD | *** | ns | ns |
| GSA | *** | * | ** |
| TRL | *** | * | ** |
| PR:S-L | ns | *** | ns |
| AngleB | * | *** | ns |
| HGI | ns | *** | ns |
| S-PR | ** | *** | ns |
| VGI | ** | *** | ns |
| CHA | *** | ns | ns |
| CHW | *** | *** | ns |
| CHH | ns | *** | ns |

ns, not significant. * $P$<0.05; ** $P$<0.01; *** $P$<0.001.

**Table 6** Correlation coefficients between the six accessions with (WW) and without (D) watering

| | CNR-No. | CNR-Lsum | CNR-TOE | LR-L | TRL | CHA | CHW |
|---|---|---|---|---|---|---|---|
| CNR-No. | 1 | | | | | | |
| CNR-Lsum | **0.85* (WW)**<br>**0.91* (D)** | 1 | | | | | |
| CNR-TOE | -0.62 (WW)<br>**-0.98*** (D**) | -0.58 (WW)<br>**-0.89*(D)** | 1 | | | | |
| LR-L | -0.17 (WW)<br>-0.68 (D) | 0.24 (WW)<br>-0.36 (D) | 0.13 (WW)<br>0.66 (D) | 1 | | | |
| TRL | 0.38 (WW)<br>**0.91* (D)** | 0.54 (WW)<br>**0.95**(D)** | -0.09 (WW)<br>**-0.92**(D)** | -0.17 (WW)<br>-0.44 (D) | 1 | | |
| CHA | 0.04 (WW)<br>0.02 (D) | 0.20 (WW)<br>0.23 (D) | -0.16 (WW)<br>-0.10 (D) | **0.82* (WW)**<br>0.21 (D) | -0.42 (WW)<br>0.42 (D) | 1 | |
| CHW | 0.17 (WW)<br>-0.18 (D) | 0.25 (WW)<br>0.00 (D) | -0.06 (WW)<br>0.10 (D) | 0.77 (WW)<br>0.25 (D) | -0.45 (WW)<br>0.21 (D) | **0.95**(WW)**<br>0.**97**(D)** | 1 |

Significant correlations are written in bold. * $P$<0.05; ** $P$<0.01; *** $P$<0.001.

**Table 7** Changes in values of root parameters of plants grown under water-limited conditions compared to the control ones

| Gaz-6 (1) | Bd18-1 (2) | Bd3-1 (3) | Bd21 (4) | Bd1-1 (5) | Bd21-3 (6) |
|---|---|---|---|---|---|
| **PR** | | | | GR:+17%*<br>L:+14%* | GR:+28%***<br>L:+21%** |
| **CNR** | No.:-100%***<br>GR:-100%***<br>Lsum:-100%***<br>TOE:+100%*** | | | No.:-30%*<br>GR:-22%*<br>Lsum:-47%** | |
| **LNR** | | No.:+500%* | No.:-75%*** | | No.:-67%** |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | GR:+1189%* | GR:-90%**<br>Lsum:-93%**<br>TOE:+14%* | | Lsum:-91%**<br>TOE:+22%** |
| **LR** | No.:-54%**<br>Lsum:-59%**<br>TOE:+28%* | | No.:+125%***<br>L:-45%** | No.:-36%**<br>Lsum:-41%** | L:-28%* | |
| **Other traits** | LRD:-58%***<br>TRL:-35%**<br>CHH:+15%*<br>PR:S-L:+20%** | angleB:-61%*<br>HGI:-57%*<br>CHW:-23%*<br>VGI:+18%* | LRD:+130%***<br>GSA:+18%*** | LRD:-26%*<br>CHA:-29%*<br>CHW:-24%**<br>TRL:-36%***<br>S-PR:+3%* | PR:S-L:+24%* | GSA:+14%**<br>CHH:+23%**<br>PR:S-L:+21%*** |

The exact values of each parameter are summarized in Online Resource 6. Numbers in parentheses represent the cluster. * $P$<0.05; ** $P$<0.01; *** $P$<0.001.

**Figures**

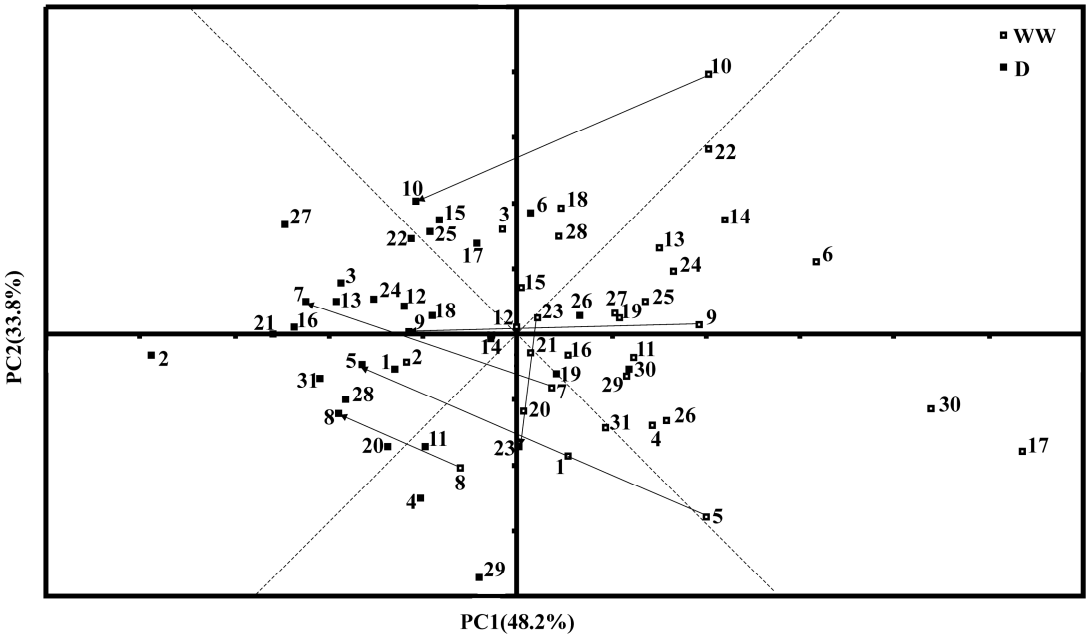


**Fig. 1** Principal component analysis of well-watered (WW) and drought stressed (D) *Brachypodium distachyon* plants. Well-watered and stressed samples of the six accessions selected for the rhizobox experiment are linked by a vector. Dashed lines represent a more descriptive direction of the traits. Squares representing genotypes are numbered according to their ID number in Table 1. Open squares indicate control samples, filled squares are samples grown during water deprivation

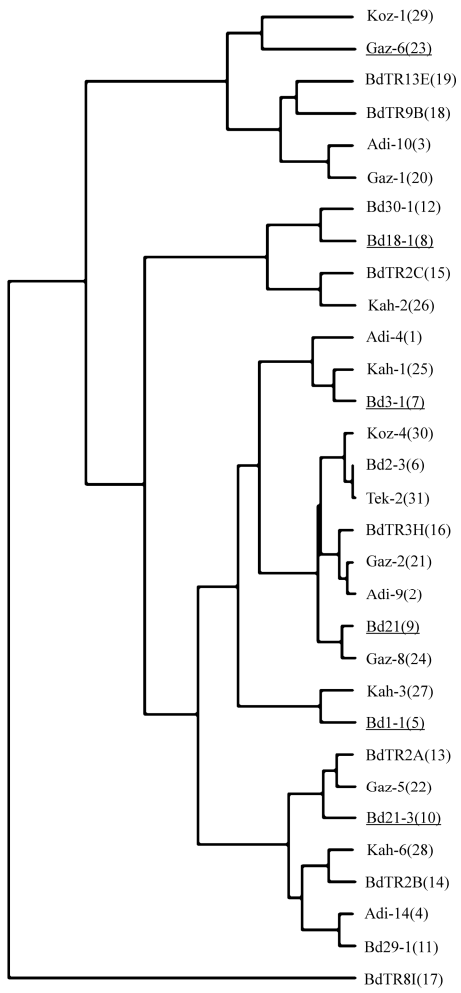


**Fig. 2** Dendrogram of the classification of the whole set of *Brachypodium distachyon* accessions, during control and water-deficit conditions. Accessions selected for rhizobox experiment are underlined

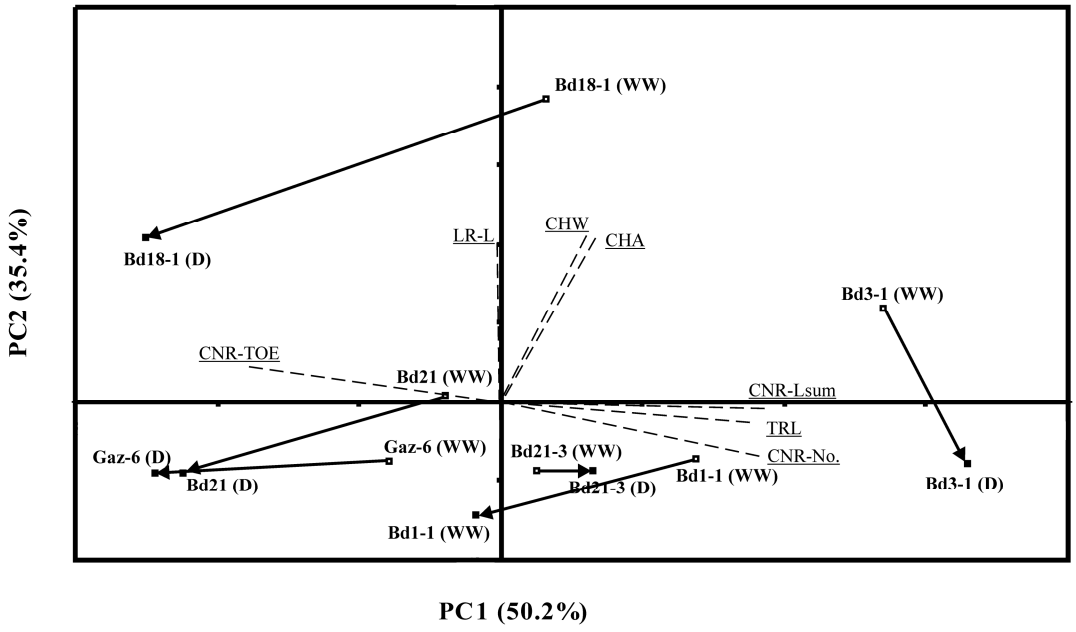


**Fig. 3** Principal component analysis of *Brachypodium distachyon* plants grown under well-watered (WW) and water-limited (D) conditions

**Annex 2 – Manuscript in preparation on improved transformation method of Brachypodium distachyon:**

Development and optimization of an efficient *Agrobacterium*-mediated transformation method in *Brachypodium distachyon*

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Abstract

The importance of monocot crop species requires a model system to study their biological mechanisms for crop improvements. The *Brachypodium distachyon* possesses similar morphological, reproductional and genetic parameters comparable to *Arabidopsis thaliana*. The existence of an established transformational methodology is also necessary. Here we report an efficient method for the transformation of *Brachypodium distachyon*. The process applies *Agrobacterium tumefaciens* infection of callus tissues. During the improvement, different *Agrobacterium* strains, selection markers, and hormone conditions were tested. The root development was fostered by 1-naphtalaneacetic acid treatment. Using this rapid method the $T_1$ progeny can be characterized and harvested within 1 year.

Keywords: *Brachypodium distachyon*, genetic transformation, *Agrobacterium tumefaciens*, 1-naphtalaneacetic acid, 6-benzylaminopurine, indole-3-acetic acid.

Introduction

The global need of the human nutrition for more food increases constantly year by year, requiring the efficient improvement of crop and forage plants in the agriculture. The majority of these plant species are originated from the Poaceae family, therefore the true grasses are indisputably the most valuable plant family for the humanity. Beside this fundamental agricultural importance they are also a predominant family amongst the monocots, thus understanding the biological processes connected to their harvest parameters is essential. For this purpose the establishment of a well-functioning model system is indispensable. The *Arabidopsis thaliana* is an ideal model plant due to its excellent parameters, however the basic structural and developmental differences

between monocots and dicots requires a monocot model plant possessing similar properties. The *Brachypodium* genus is located at the base of the four grass tribes that includes the majority of domesticated cereal and forage crops (Kellogg 2001), and taken together with the grass genome's remarkable colinearity, as well as the other prosperous parameters of the *Brachypodium distachyon* – the single annual species from this genus – , such as the small, diploid, sequenced genome (International Brachypodium Initiative 2010), short, up to 12 weeks long generation time, small plant size, simple growing conditions and self-pollination, this plant seems to be the ideal experimental model plant for the Poaceae. To obtain this state a well-established transformational method is essential, as well. However, previously both of the most prevalent methods – microprojectile bombardment (Christiansen et al.) and the *Agrobacterium*-mediated (Vogel et al.) – were described in *Brachypodium distachyon*, in this study the later was applied due to its obvious advantages appearing in the reduced possibility of the rearrangement and fracture of the transported DNA fragment, and in the lower copy number of the transgene. Considering that there is no well-working *in planta* transformational process described in *Brachypodium*, tissue culture was applied. The effectiveness of different plant selection markers and *Agrobacterium tumefaciens* strains were also tested during the improvement of the process. Beside the introduction of the desired DNA fragment the most important and crucial step is the regeneration of the transformed callus pieces during an *in vitro* culturing process. In this study, along the widely used kinetin, 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA) were applied during the regeneration steps. To obtain sufficient amount of healthy plantlets that are able to cope with the greenhouse conditions as *in vitro* plants, the proper root induction and strong root development is an absolute necessity. Employing the common protocols the occurrence of *Brachypodium* plants growing weak, underdeveloped root system is rather frequent according to our observations. The regenerating plants refusing to develop sufficient root system were fostered to proceed by naphthalene acetic acid (NAA) treatment, resulted in 100% survival rate after potting in the greenhouse.

## Materials and Methods

### Agrobacterium tumefaciens *strains, plant material and callus initiation*

The LBA4404 and the supervirulent AGL-1 *Agrobacterium tumefaciens* strains were used in the experiments. The plasmids transferred into the bacteria were the pCXGL13p and the pEGAD, containing different plant selection markers; hygromycin phosphotransferase and phosphinothricin acetyltransferase, respectively (Fig. 1). 100 ng of the plasmids were introduced into electrocompetent cells by electroporation (2.2 kV). The success of the transformation was verified by colony PCR and back-transformation of plasmid minipreps from the PCR-positive colonies into *Eserichia coli* cells, followed by the digestion with the appropriate restriction endonucleases of the *E. coli* derived plasmid minipreps.

The *Brachypodium distachyon* Bd21 inbred line was used the *in vitro* culture experiments. The embryo donor plants were grown in greenhouse at 24°C with supplemented lighting extended to 18 h daylight. The seeds were collected 20 days after visible anthesis, surface sterilized by washing in 10% household bleach for 5 min. The seeds were then rinsed three times with sterile distilled water. The immature embryos were excised under microscope in a laminar flow box and put immediately onto LS/R medium (basal LS salts plus 40 g/l sucrose and 7 g/l plant agar, pH 5.8) containing 2.5 mg/l 2,4-D with scutellar side down. Then the embryos were incubated in the dark at 24°C. After 2 weeks the developing root and shoot organs were eliminated, and only the yellowish, solid embryonic calli pieces were sub-cultured onto the same medium.

*Plant transformation*

2 weeks after subculturing, the calli were harvested from the plates, chopped into pieces (cca. 1 mm in diameter) with sterilized razorblade, and put into R2/MED liquid medium (basal R2 salts plus 10 g/l glucose, pH 5.2) containing 2.5 mg/l 2,4-D, 100 µM acetosyringone and 500 µl *Agrobacterium* inoculum (OD600= 0.8) into a Büchner flask. After 5 min of vacuum treatment, the flasks were placed onto a shaker for 1 h shaking at 100 rpm at room temperature. The calli were taken out, dried using sterile filter paper disks, and transferred into Petri dishes containing R2/MED medium (as above plus 7 g/l plant agar, pH 5.2) with 2.5 mg/l 2,4-D, 100 µM acetosyringone for co-cultivation. The plates were kept 1, 2 or 3 days in dark at 24°C, then the calli were harvested, and washed by shaking at 100 rpm for 30 min in the liquid R2/MED medium containing 1 g/l Augmentin, 300 mg/l Timentin to eliminate the Agrobacterium. The calli were dried on sterile filter paper and put into Petri dishes containing R2/S medium (basal R2 salts plus 30 g/l sucrose, pH 6.0) with 2.5 mg/l 2,4-D, 200 mg/l Augmentin, 150mg/l Timentin, 40 mg/l hygromycin B or 6, 8, 12 mg/ml phosphinothricin (PPT), and kept in dark at room temperature. After selection, the yellowish calli were put onto fresh R2/S media supplemented with the same concentration of hygromycin B, or PPT and Timentin as previously. The overgrowing yellow, solid calli were then put onto embryogenecity improvement media (LS/E: basal R2 media containing 30 g/l sucrose, 2.5 mg/l 2,4-D, 50 mg/l hygromycin B, 150 mg/l Timentin, pH=5.8). After 10 days samples from the calli were taken for DNA isolation in order to verify the genetic transformation.

*Plant regeneration*

The calli were put in Petri dishes containing LS/R medium supplied with 0.5 mg/l indole-3-acetic acid (IAA) and 0.3 mg/l 6-benzylaminopurine (BAP), or 0.2 mg/l kinetin, and placed in light conditions at 24°C. The regenerating plantlets with underdeveloped roots were replaced into jars or glass tubes containing 0.5xMS medium (half of the basal MS salts plus 10 g/l sucrose and 2.5 g/l gerlit, pH 5.8) supplied with 50 µg/l 1-

naphthaleneacetic acid (NAA). The plants grown well-developed root system were planted in the greenhouse.

*Genomic DNA isolation, PCR*

100 mg frozen callus tissue was ground in liquid nitrogen for the genomic DNA isolation. The DNA was extracted using a modified CTAB method.

The primers used in the polymerase chain reactions were designed with the online software PrimerQuest at the IDTDNA website (www.idtdna.com). For the analyses of the candidate plants transformed using the pCXL13p plasmid, the forward primer (35SF: CGCACAATCCCACTATCCTT) hybridized with CaMV 35S promoter sequence, the reverse primer (PHYGR: GCTCATCGAGAGCCTGC) hybridized with the hygromycin phosphotransferase gene. In the case of the transformation using the pEGAD vector, both the forward (GFPF: CGACAAGCAGAAGAACGGCATCA) and the reverse primer (GFPR: GGCGGTCACGAACTCCAGCA) hybridized with the GFP reporter gene. The PCR products were analyzed by standard agarose gel electrophoresis.

## Results and Discussion

For the *in vitro* culture experiments the Bd21 inbred line was selected, because this is the genotype which sequenced genome is available, and this ecotype tolerates well the stress caused by tissue culturing (Vogel et al. 2006). The distribution of the embryo size and the state of its development was high, even though the spikes were harvested all at once, 20 days after pollination. The size of the excised embryo is an important feature, it can determine the rate of the developed embryogenic calli, which is the source of the transformation material; the smaller embryos (<0.5 mm in diameter) produce better quality callus tissues. Generally the 90% of the isolated immature embryos produced calli tissues successfully. After sub-culturing, the calli were chopped to gain more transformable material. This treatment did not harm the development of the *in vitro* plants as it was proved by a preliminary experiment, when we were able to regenerate wild type *Brachypodium distachyon* plants from equally treated callus material.

The application of the two variant *Agrobacterium tumefaciens* strain (LBA4404 and AGL-1) exhibited significant differences in the transformational efficiency. Previously the LBA4404 strain was used successfully for genetic transformation of barley scutellums, but only two independent callus lines were positively tested by genomic PCR. In contrast, the transformation using the AGL-1 strain increased the number of the independent transformed lines up to 115. For the selection of the candidate callus lines, the hygromycin B in 50 mg/l concentration appeared to be the better option. In the case of phosphinothricin (PPT) selection defining the selective concentration was difficult, the best results were obtained using 8 mg/l, but still several non-transformed surviving callus lines could be found. The experiments for the optimization of the length of the co-cultivation time revealed that the sufficient duration is two days long. Shorter co-cultivation resulted in significantly decreased transformational efficiency, while the extended treatment caused the overgrowth of the *Agrobacterium* culture and made it

difficult to eliminate, even with additional washing using combined antibiotic solutions (amoxicillin, ticarcillin and clavulanate). Thus, during the most productive transformational event AGL-1 *Agrobacterium* strain was used, which harbored a plant expression vector that contained hygromycin phosphotransferase plant selection marker, and co-cultivated for two days in the dark with the callus culture.

In the first regeneration steps 0.2 mg/l kinetin was applied in order to induce the differentiation of the callus tissues, which is widely used according to the literature (Vogel et al., Christiansen et al., Pacurar et al.). Our finding was that the most of the calli developed shoots, but the root formation was insufficient or completely missing rather frequently. Obviously, cytokinins induce the growth of lateral buds and the cancellation of their dormancy. In addition, they play pivotal role in cell cycle regulation. Similarly, auxins influence the initiation of cell division, and in the other hand they participate in the cell differentiation processes. Higher auxin level stimulates cell division, while low auxin concentration drives cell elongation, cell enlargement, and cell differentiation. (Winicur 1998, Zazimalova 1995). They also play role in cell expansion, promotion of vascular development via induction of the phloem and xylem differentiation, and the formation of the root system (Gaspar et al. 1996), therefore the tuning of the auxin:cytokinin balance could be beneficial in the manipulation of the developmental and processes of the *in vitro* plants, because it is known that their balance can orient an organogenic program (Gaspar et al. 2003). Auxin and cytokinin ratio can be manipulated to promote proliferative callus growth, regeneration of vegetative tissue, or root induction. Elevated auxin/cytokinin ratio leads to root formation, low ratio facilitates buds regeneration (Perrot-Rechenmann and Napier, 2005). So to facilitate the root development we changed the exclusive supplement of cytokinins (kinetin) to a cytokinin:auxin mixture, which contained the most common auxin indole-3-acetic acid in 0.5 mg/l concentration and 0.3 mg/l of 6-benzylaminopurine which is a first generation synthetic cytokinin. This modified regeneration media triggered the root formation in the 55% of the rootless lines 2 weeks after the passage of the original calli. However, in most of the cases the shoots` development was shown to be more advanced than the roots`, therefore the plants might not be strong enough to survive the stress that the potting of an *in vitro* plant in the greenhouse can create. In order to strengthen the root system, the developing brome plants were replaced into glass tubes containing MS medium supplied with 50 µg/l naphtalaneacetic-acid (NAA) synthetic auxin. NAA is a well-known rooting agent, which reportedly is able to increase the number of root primordia in monocots rice (Zhou, 2003), black rush (Wang, 2005), *Gladiolus* (Ghani, 2008), or in dicots tomato (Khan, 2011) and lettuce (MacIsaac, 2006). This effect is inhibited by kinetin. Also, to foster the root penetration into the media, the concentration of the plant agar was decreased by 50% that made the media suitable for the extension of the roots. After two weeks the plants developed strong, thick roots, made them prepared for planting. To reveal the optimal time for this treatment, the half of the plants were kept in this environment for two more weeks. These plants grew much stronger root system, however due to the short lifespan of the *Brachypodium* they became over-matured for potting, the majority started to develop spikelets, and they grew to the half size in the greenhouse compared to the plants spent less time in glass tubes, producing significantly decreased amount of seeds as well.

The described transformation methodology is a rapid and efficient process. The $T_0$ seeds can be harvested in 30 weeks, and allows the characterization of the $T_1$ progeny and the collection of the $T_1$ seeds within one year. These data reflects also the relevancy and applicability of the *Brachypodium distachyon* as a suitable model plant for the monocot crop plants.

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Figures

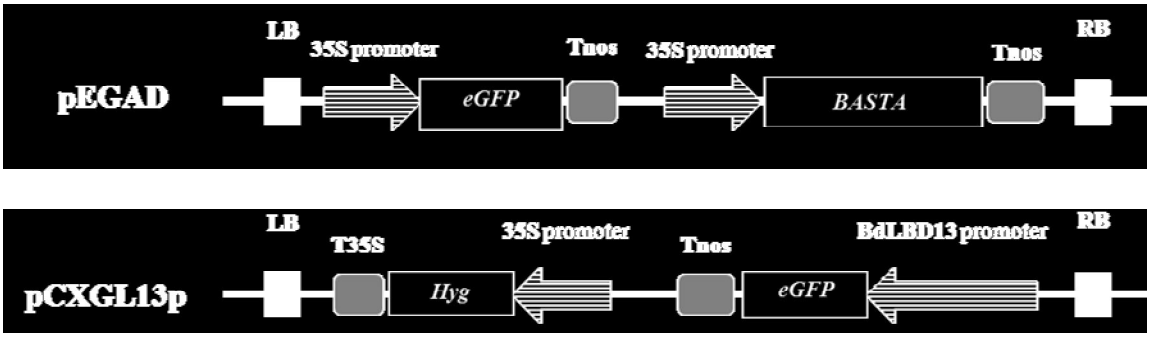


Fig. 1. The vectors used throughout the optimization experiments. LB: Left border sequence, RB: right border sequence, 35S promoter: promoter of the CaMV 35S RNA, BdLBD13 promoter: promoter of the *Brachypodium distachyon* LBD13 gene, T35S: terminator sequence of the CaMV 35S RNA, Tnos: terminator sequence of the nopaline synthase, BASTA: phosphinothricin acetyltransferase gene, Hyg: hygromycin phosphotransferase gene.

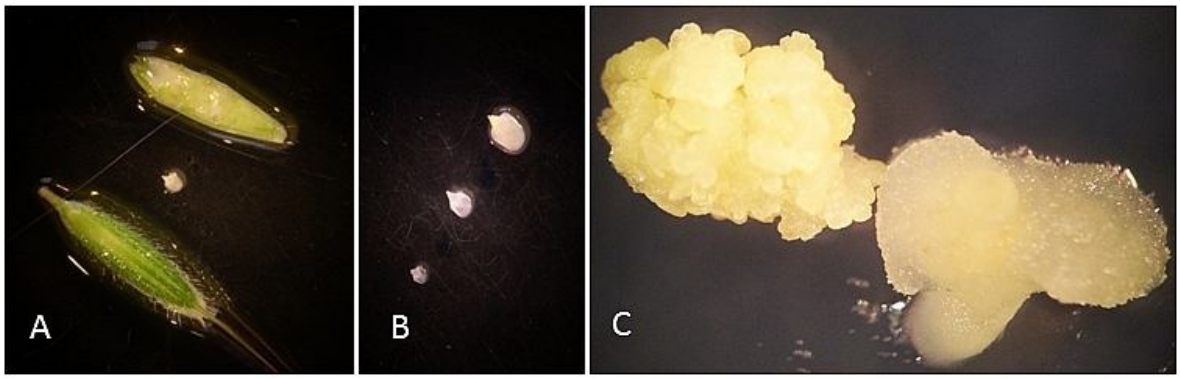


Fig. 2. Embryo isolation and callus induction. A: Isolated embryo and seeds. B: size distribution of different embyos. C: Callus tissues developed after 3 weeks of the induction. The left piece is a suitable material for the transformation.

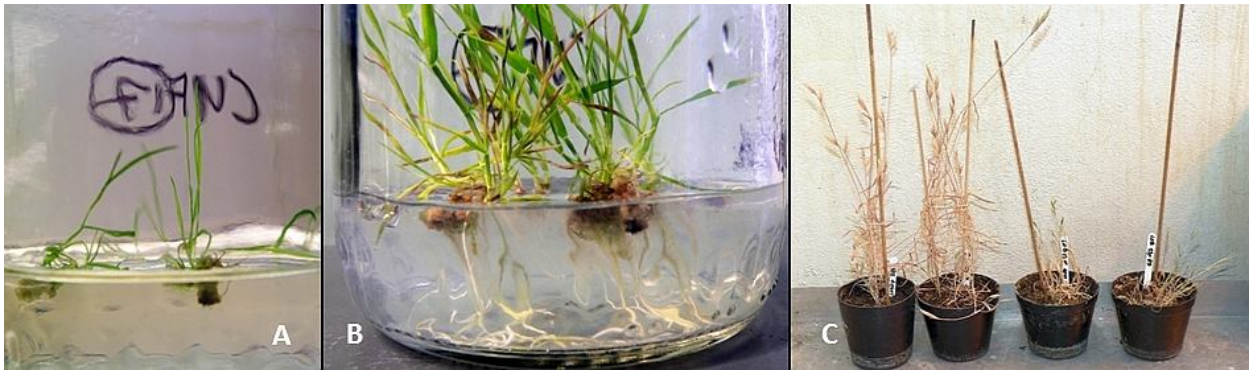


Fig. 3. The effect of the naphthaleneacetic-acid treatment. A and B: regenerating brome plants before and after the 50 µg/l NAA application. C: the consequence of the long *in vitro* culture.