EFFECT OF EXOGENOUS APPLICATION OF 2,4-D ON *MEDICAGO TRUNCATULA* PLANTS WITH MODIFIED AUXIN TRANSPORT IN EXTREME PHOSPHATE CONDITIONS

Miglena Revalska, Anelia Iantcheva

AgroBioInstitute, Blvd. Dragan Tzankov 8, 1164 Sofia, Bulgaria

Abstract

2,4-Dichlorphenoxyacetic acid (2,4-D) is a synthetic auxin analogue, mainly used as a herbicide that is absorbed through the leaves and translocate in the plant. It is a substrate for LAX-transporters and about 75% thereof requires "influx" carrier to enter the cell, especially if its concentration outside the cell is low. Phosphorus is one of the most important macronutrients required for normal development of plants. The phytohormones auxin and strigolactone participate in the plant response associated with the phosphate signalling. The aim of the study was to monitor the effect and influence of exogenous application of 2,4-D on the growth and development of root system and expression level of the analysed MtLAX3 gene in extreme phosphate conditions. For this purpose, seeds from T_1 Medicago truncatula plants with modified auxin transport and wild type plants, were germinated and subsequently grown in phosphate deprivation or excess conditions, and additionally treated with 2,4-D. Furthermore, relative transcript level of the strigolactone gene MtMAX2 was examined in the same plants to reveal the interaction of auxin and strigolactones in these conditions. The expression of GUS reporter gene fused to MtLAX3 promoter was traced in different organs and tissues by histochemical GUS assay before and after application of exogenous 2,4-D.

Key words: *Medicago truncatula, auxin, strigolactone, 2,4-D, phosphate deprivation, MtLAX3*

1. INTRODUCTION

In plants, the control of many developmental processes is maintained by hormone interactions (Foo 2012). Phytohormones are small signaling molecules that play crucial role during all stages of plant growth and development. Hormonal crosstalk between auxin and strigolactones regulate plant development and can fine-tune the root system (Ruyter-Spira et al. 2011; Cheng, Ruyter-Spira & Bouwmeester 2013; Rasmussen et al. 2013). Both phytohormones are involved in the control of shoot branching (Beveridge & Kyozuka 2010), in the regulation of arbuscular mycorrhizal symbiosis (Foo 2012), regulation of bud outgrowth (Brewer et al. 2009), secondary growth and root hair elongation (Kapulnik et al. 2012). Strigolactones may regulate the local levels of auxin in plants, either by changing the biosynthesis of auxin or polar auxin transport (Ruyter-Spira et al. 2011; Rasmussen et al. 2013; Shinohara, Taylor & Leyser 2013). Auxin and strigolactones has been shown to participate in the response of plants associated with the phosphate signaling and the response to low phosphate (Mayzlish-Gati et al. 2012; Peret et al. 2014). According to Mayzlish-Gati et al. (2012), under conditions of Pi depletion *MAX2* activate TIR1 expression and auxin signaling pathway. In our previous study, *Medicago truncatula* transgenic lines overexpressing *MtLAX3* were characterized by high plasticity of surviving phosphorus starvation due to the significant increase in lateral roots number and the relative expression level of *MtLAX3* (involved in polar auxin transport), *MtMAX2* (involved in strigolactone signaling) and *MtMAX3* (involved in strigolactone biosinthesis) (Revalska & Iantcheva 2018). Consequently, we might assume that *MtLAX3* activates phosphate cascade by transporting auxin through the cell membrane into the cell. Most likely phosphorus deficiency activates the function of *MtLAX3* and consequently stimulates secondary rooting and inhibition of main root growth. Our results confirmed previous studies about concert act between strigolactone, auxin, citokynin and other plant hormones where the central role is played by the capacity of polar auxin transport (Jiang et al. 2015; Maathys et al. 2016) in shaping the root architecture. These results help to understand *MtLAX3* function in legumes and can be used to optimize the growth and development of important legumes in eroded and poor of phosphate soils. In conditions of phosphate

deficiency in plants many secondary roots are formed, which is of importance for improving the physicochemical parameters of the soil. The main objective of this research was to study the effect of exogenous application of the synthetic auxin analogue 2,4-D (2,4-Dichlorphenoxyacetic acid) on *M. truncatula* plants with modified auxin transport (overexpression-OE and downregulation-RNAi of *MtLAX3* gene) and wild type (WT) placed in extreme conditions of phosphate deprivation or excess. In general, 2,4-D is mainly used as a herbicide that is absorbed through the leaves and translocated in the plants (Simon & Petrasek 2011). In our previous study we determined that MtLAX3-OE plants are able to mitigate the effect of Pi-starvation and confirmed the interaction between auxin and strigolactones (Revalska & Iantcheva 2018). For the past few years the subject of our research was *MtLAX3* gene, initially identified by a reverse genetic approach in a population of *Tnt1* retrotransposon-tagged mutants of *M. truncatula* (Revalska et al. 2011). *Tnt1*-insertion mutant collections for *M. truncatula* was generated and disrupted genes by the *Tnt 1* retrotransposon were identified (d'Erfurth et al. 2003; Tadege, Ratet & Mysore 2008; Iantcheva et al. 2009). The flanking sequence tag (FST) information has been generated for many of the lines and deposited for public use at the Samuel Roberts Noble Foundation (http://bioinfo4.noble.org/ mutant/). The FSTs of one of the *Tnt1 inserts* partially correspond to the gene encoding an Auxin influx carrier protein, *LAX3*, which isinvolved in polar auxin transport (Noorden et al. 2006; Revalska et al. 2015).

The seeds were collected and subsequently sterilized by using previously described method (Revalska et al. 2015). Afterwards homozygous seeds of OE and RNAi-lines, *MtLAX3* transcriptional reporters and WT plants were cultivated on medium with low, high and normal content of Pi for 14 days. All of the germinated seedlings continued their growth at the same content of Pi and additionally were treated with 0,1 µM 2,4-D for another 7 days. In our previous studies (Revalska et al. 2015; Revalska & Iantcheva 2018) we have explained in details how the T_1 transgenic lines used for this experimental set were selected. We used 3 independent T_1 transgenic lines for every construct for the phenotypic, morphometric and expression analyses. For performing histochemical GUS assay, the expression of *GUS* reporter gene fused to *MtLAX3* promoter was traced in different organs and tissues in 3 different T_1 lines. The experimental set includes phenotypic analyses of the root architecture of OE and RNAi-lines and WT before and after 2,4-D treatment; measurement of the main root length (MRL) and lateral root number (LRN) in OE and RNAi-lines and WT before and after 2,4-D treatment; transcriptional analysis of *MtLAX3* and *MtMAX2* before and after treatment in different plant organs (leaves and roots); and histochemical GUS assay of *MtLAX3* promoter after exogenous application of 2,4-D.

As we know, both auxin and strigolactones are involved in phosphate signaling and 2,4-D is a substrate for LAX-transporters we assumed, that 2,4-D will act as an activator of the auxin signaling pathway, and thereby will activate the strigolactone gene *MAX2*. This will lead to slight elongation of the main root and very drastic increase in LRN, as well as up-regulation in the relative transcript level ot the two investigated genes – *MtLAX3* and *MtMAX*2.

2. MATERIALS AND METHODS

2.1. Plant material and plant growth

To accomplish the experimental work WT seeds from *M. truncatula* cv. 'Jemalong 2HA' (Nolan, Rose & Gorst 1989) and T_1 seeds from transgenic plants with OE and downregulation of *MtLAX3*, as well as *MtLAX3* transcriptional reporters were used. The production of *MtLAX3* transgenic plants, together with the cloning work and plasmid construction were described in details in Revalska et al. (2015). For each transgenic construct (OE, RNAi and transcriptional reporter) three independent lines were used. The seeds were scarified, surface sterilized with ethanol - 70% (v/v) and mercury chloride ($HgCl_2$) - 0.1% (v/v), than washed several times with sterile distilled water (Revalska et al. 2015). According to Revalska & Iantcheva (2018), the seeds were stacked on a sterile filter paper (Whatman) and placed in dark for 48 h at 4 °C. Then pool from 15 seeds of every transgenic line of each construct (OE, RNAi and transcriptional reporter) were placed in square Petri dishes on ½MS medium supplemented with KH_2PO_4 (as a source of Pi) in different concentrations: high − 2 mM (272 mg l^{-1}),

low − 1 μM (0.136 mg l^{-1}) and normal 0,63 mM (85 mg l^{-1}, which is the content of the phosphate salt in the basic MS_0 culture medium). Petri dishes were placed vertically in the cultivation room and T_1 seedling progeny was grown for 14 days post germination (dpg) under *in vitro* conditions at constant maintains of these indicators: temperature 23–24 °C, constant relative humidity, photoperiod 16/8 h (day/night), light intensity 150–300 mol m^{-2} s^{-1}. Germinated seedlings continued their growth at the same content of Pi and additionally were treated with 0,1 μM auxin 2,4-D for another 7 days.

2.2. Phenotypic analyses and biometric measurements

Phenotypic characterization of the root system of the transgenic (OE and RNAi) and WT plants was done by using stereomicroscope Nikon G-AL, equipped with camera. The main root length (MRL) was measured and lateral root numbers (LRN) were counted at the 14th dpg and then in the 21st dpg, after 7 days treatment with 2,4-D. The measurements were performed with the software program ImageJ v.1.4.3.

2.3. RNAi-isolation and Real-Time quantitative reverse transcription PCR (qRT-PCR)

Samples from leaves and roots of 3 independent lines of OE/RNAi and WT plants grown for 14 days under low, high and normal Pi conditions were collected by rapid freezing with liquid nitrogen. Subsequently, leaves and roots samples from same transgenic and WT plants, grown for another 7 days on the same media supplemented with 2,4-D were collected in the same way. Total RNA was isolated by using EurEx with RNeasy Plant Mini Kit. One Step SYBR® PrimeScript™ RT-PCR Kit II (TaKaRa BIO INC.) was used for the real-time quantitative reverse transcription reaction. The relative transcript level of *MtLAX3* and *MtMAX2* were determined with by using the standard protocol for Applied Biosystems 7300 Real-Time PCR System. For data normalization the housekeeping genes actin (*ACT*) and ubiquitin (*UBQ10*) were used. Primers used for qRT-PCR analysis are given in Table 1.

F-Mt-actin	5'-TCAATGTGCCTGCCATGTATGT-3'
R-Mt-actin	5'-ACTCACACCGTCACCAGAATCC-3'
F-Mt-Ubiquitin	5'-GCAGATAGACACGCTGGGA-3'
R-Mt-Ubiquitin	5'-AACTCTTGGGCAGGCAATAA-3'
F-Mt-LAX3 MT3G072870	5'-GCACTCTGGTCCAACCAAAT-3'
R-Mt-LAX3 MT3G072870	5'-AGGGCATTGGAATGAGTGAG-3'
F-MtMAX2	5'-ACTATCCAGCACCAGAGAATGAC-3'
R-MtMAX2	5'-GACGCCTATTCAACGCATCTTC-3'

Table 1. Genetic sequences of primers used for qRT-PCR analysis

2.4. GUS staining

T_1 seeds from *MtLAX3* transcriptional reporter plants of *M. truncatula* were germinated and subsequently grown as described in *2.1. Plant material and plant growth*. At the 14th and the 21st day samples were collected and stained in GUS solution by using protocol described in Revalska et al. (2015). The different parts from the seedlings were photographed by using stereomicroscope Nikon G-AL, equipped with camera.

2.5. Statistical analysis

All of the experiments in this study were repeated three times and triplicate assays were performed for each experimental data set. The software program qBase v1.3.5 was used for analyzing the data from qRT-PCR. The results were expressed as mean ± standard error mean (SEM). Phenotypic data and morphometric data were analyzed with Two-Way ANOVA from the software program OriginPro

v.8.5.1 as an appropriate method to analyze the main effects of and interactions between two factors - genotype and treatment. The *p* values were considered to be statistically significant at 0.05 levels. As a means comparison method, Tukey test was used, where sig equals 1 indicates that the difference of the mean is significant at the 0.05 level. Sig equals 0 indicates that the difference of the mean is not significant at the 0.05 level. The results are expressed as the mean ± standard deviation and OriginPro v8.5.1 software was used to create the charts for qRT-PCR and phenotypic analysis. The differences were assessed by t-test as well and considered statistically significant at $p < 0.05$.

3. RESULTS

In this research we determined the effect of exogenous application of 2,4-D on *M. truncatula* plants with modified auxin transport after Pi-starvation/excess on root phenotype and architecture. The expression level of the auxin and strigolactone genes studied was measured before and after 2,4-D application. For this purpose we have grown pool from T_1 seeds of 3 independent transgenic lines from *M. truncatula* plants with MtLAX3-OE, RNAi and transcriptional reporters as well as WT plants, on MS_0 or basal medium supplemented with 1 µM (deficiency) or 2 mM (excess) of Pi for the time period of 14 days. Afterwards same lines continue their growth on the same media supplemented with 2,4-D. Three independent lines from every transgenic construct were investigated and the results obtained were average.

3.1. Phenotypic analysis and evaluation of the MRL and LRN of M. truncatula plants with modified auxin transport and wild type

MtLAX3-OE, MtLAX3-RNAi-lines and WT (2HA control plants) were grown *in vitro* for 14 days in MS medium supplemented with different content of Pi: MS_0 – basal MS medium; 1 µM – MS medium supplemented with 1 µM Pi; 2 mM – MS medium supplemented with 2 mM Pi (Figure 1) Phenotypic analysis, together with biometric measurements of the main root length (MRL) and lateral root number (LRN) were performed. At the 14 dpg, the transgenic and WT plants were moved to the same media supplemented with 0,1 µM 2,4-D and subsequently grown for another 7 days (MS_0 +2,4-D; 1 µM+2,4-D; 2mM+2,4-D) (Figure 1). Phenotypic analysis of the root architecture and biometric measurements of the MRL and LRN were performed 21 dpg (Figure 2 and Figure 3).

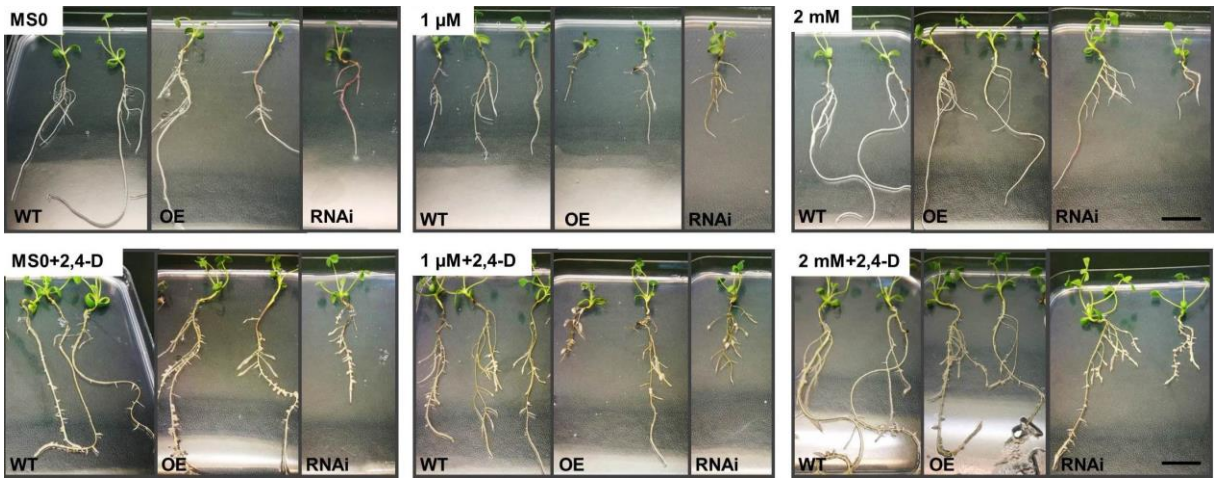

Figure 1. Morphological and phenotypic evaluation of the root system of T_1 *M. truncatula* OE and RNAi lines, and WT plants grown in different phosphate conditions before and after 2,4-D treatment. Scale bar = 5 cm.

In basal MS_0 medium OE-lines possessed a long primary root with short multiple secondary branches, similar to the phenotype observed in 2HA control. In RNAi plants, we noticed slower growth and

shorter root system with very few and thin lateral roots (LR). In Pi-deficient medium (1 µM Pi) we monitored alike root phenotype in the transgenic and WT plants, to wit short main root with many LR, in comparison with plants grown in normal conditions (MS_0, not treated plants). In general OE and RNAi-lines possess less LRN than WT plants (Figure 1). RNAi-lines grown on Pi-deprivation medium possess brownish roots due to phosphate deprivation. In *M. truncatula* WT plants and transgenic lines the excess of Pi in the medium (2 mM) induced the formation of extended and branched root (Figure 1). RNAi-lines were characterized with similar phenotype as WT plants and OE-lines when Pi in the medium was increased, but the root system was better developed than in RNAi-lines grown in normal conditions.

After 2,4-D treatment, we monitored formation of many more LR in WT and transgenic lines in normal and extreme conditions of Pi (Figure 1). In MS0+2,4-D conditions WT and OE plants possess long MR with many more LR and lots of root hair, compare to plants non-treated with 2,4-D. The root of RNAi lines remained short but the number of lateral roots, covered with root hairs, was increased. Plants grown in Pi-deficient medium supplemented with 2,4-D remained with similar MRL as non-treated with 2,4-D plants, but with increased LRN. The lateral roots were characterized with fluffy ends. OE and RNAi-lines grown in 2 mM Pi+2,4-D possess increased LRN in comparison with non-treated with 2,4-D plants grown in 2 mM Pi. WT plants developed less LRs than OE and RNAi lines in the same conditions, but more LRs in comparison with WT plants non treated with 2,4-D (Figure 1).

To be able to confirm the results obtained by phenotypic analyses, we evaluated the root system of the transgenic lines and WT plants by two parameters – MRL and LRN. In Figure 2 and Figure 3 was shown how the genotype and treatment influence the MRL and LRN. MRL and LRN were measured in 2 time points – 14th and 21st days post germination (dpg). From the 14th to the 21st day, to the medium with different content of Pi was added 0,1 µM 2,4-D.

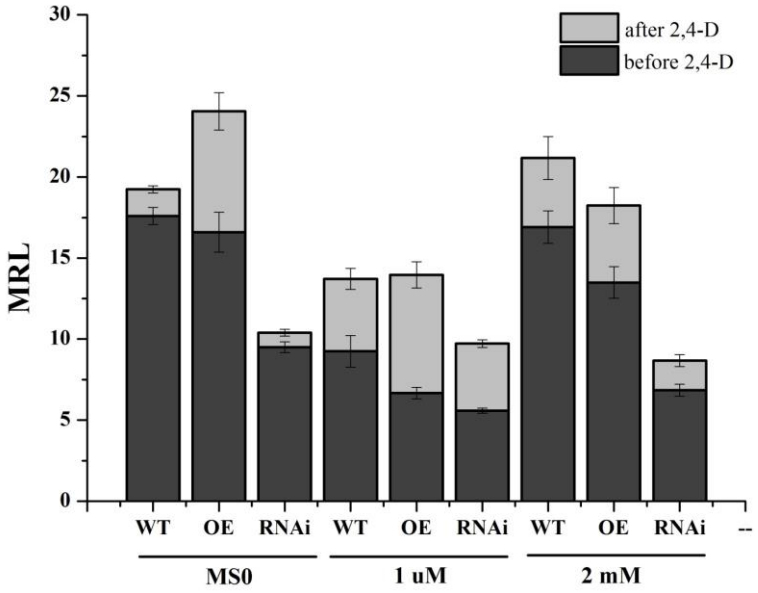

Figure 2. Biometric evaluation of main root length (MRL in cm) in T_1 *M. truncatula* OE and RNAi-lines, and WT plants grown in different phosphate conditions, before and after 2,4-D treatment.

Before 2,4-D treatment, in MS_0 conditions MRL of OE-lines (Figure 2) was significantly increased compared to RNAi-lines ($p<0.001$) and not significantly decreased compared to WT plants ($p>0.05$). Pi-deprivation in the medium led to significant reduction of MRL in OE and RNAi-lines compared to WT ($p<0.001$), but no significant difference was found between OE and RNAi-lines ($P>0.05$) (Figure 2). 2 mM Pi in the medium led to significant elongation in the main root of WT plants (3-fold) and OE-lines (2-fold) compared to RNAi-lines ($p<0.001$; $p<0.01$). The main root in WT was longer than in

OE-lines ($p<0.01$). In general, Pi-deprivation caused significant reduction in MRL in the transgenic and WT plants for 14 days cultivation in comparison with the plants grown in MS_0 and 2 mM Pi, where the results for MRL were similar.

In normal MS_0 conditions, treatment with 2,4-D significantly stimulate the elongation of main root in OE-lines, compared to WT ($p<0.01$) and RNAi-lines (0.01). In the conditions of low Pi concentration MRL in OE-lines was 2-fold increased, compare to RNAi-lines and WT plants ($p<0.01$). The MRL in OE-lines and WT was comparable in excess of Pi and 2.5-fold longer than in RNAi-lines ($p<0.01$; $p<0.01$). Treatment with 2,4-D led to significant elongation in MRL in the transgenic and WT plants in low Pi-conditions; in OE-lines grown in normal conditions and in OE and WT grown in 2 mM Pi. Slight elongation of the MRL was observed in RNAi-lines and WT grown in normal conditions, and in RNAi-lines in high Pi-conditions.

In Figure 3 was shown the differences of LRN in the transgenic lines and WT plants in normal, low and high Pi conditions, before and after 2,4-D treatment.

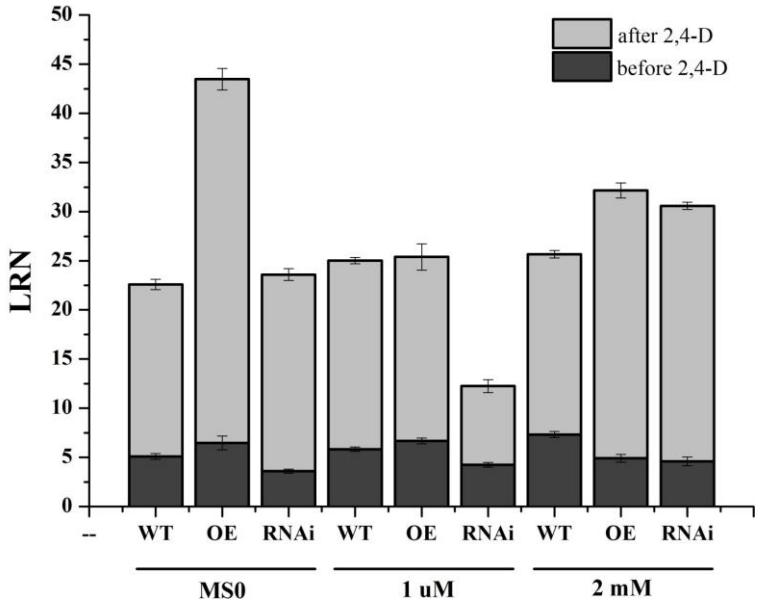

Figure 3. Evaluation of lateral root number (LRN) in T_1 *M. truncatula* OE and RNAi-lines, and WT plants grown in different phosphate conditions, before and after 2,4-D treatment.

In normal MS_0 conditions LRN in OE-lines was increase compared to WT ($p<0.01$) and almost 2-fold increase compare to RNAi-lines ($p<0.001$). For 14 days, the low Pi concentration in the medium led to similar number of lateral roots in the transgenic and WT plants as in MS_0 conditions. OE and RNAi-lines grown in high concentration of phosphate possess similar LRN ($p>0.05$), but in WT plants, LRN was increased compared to OE (1.5-times; $p<0.01$) and RNAi-lines (1.6-times; $p<0.01$) (Figure 3). For 14-days treatment with different Pi-content in the medium we did not observed many differences in the LRN, except in 2 mM Pi, where the LRN was mostly increased in WT.

On the other hand, the treatment with 2,4-D led to drastic increase of the LRN in all of the transgenic lines and WT, in the three different media (Figure 3). In MS_0+2,4-D conditions, we observed 8-times more newly developed LRN in the OE-lines, 2-times more than in WT ($p<0.01$). In the RNAi-lines LRN was slightly increase in comparison with the WT ($p=0.05$). 2,4-D treatment in Pi-depletion medium led to almost 5-fold increase in the LRN of OE and WT, but comparable with each other ($p>0.05$). In RNAi-lines LRN was increase 2,5-fold after 2,4-D treatment, but decreased to wit OE and WT ($p<0.01$; $p<0.01$). In conditions of high Pi supplemented with 2,4-D we observed 3-times more newly developed LR in WT and more than 6-fold increase in the LRN in OE and RNAi-lines. OE and

RNAi-lines possess significant difference in the number of LR compared to WT ($p<0.01$; $p<0.01$), where in the OE LRN was not significantly increase in comparison with RNAi-lines ($p>0.05$). In our experiment we observed the greatest increase in LRN of OE-lines grown in MS_0+2,4-D, and stimulation of the development of new LR in OE and RNAi-lines, as well as WT grown in 2 mM+2,4-D (Figure 3).

3.2. Assessment of the relative transcript level of MtLAX3 and MtMAX2 in M. truncatula plants with modified auxin transport and wild type

We have evaluated the transcript level of auxin influx transporter *MtLAX3* and the strigolactone gene *MtMAX2* in T_1 MtLAX3-OE and RNAi-lines from *M. truncatula*, and in WT plants, after 14-days treatment with 1 µM and 2 mM Pi, and without treatment – MS_0 normal conditions (Figure 4) in the upper part (leaves) and in the roots. Further, we measure the expression of the same genes in the same parts of the plants after additional 7 days treatment with 2,4-D (add as follows in 1 µM, 2 mM Pi and MS_0 nutritional environment).

In normal, low and high Pi-conditions *MtLAX3* expression profile between the genotypes was similar – up regulation in OE-lines and down regulation in RNAi-lines, in leaves and roots. After 2,4-D treatment up-regulation of the gene was observed in the roots, but not on leaves (Figure 4). In leaves 14 days of Pi-starvation led to increase of *MtLAX3* transcript level in OE-line, compare to normal and high phosphate conditions. In roots 2 mM Pi in the medium led to significant up-regulation of the gene in OE-lines. Highest expression of *MtLAX3* was detected in the roots of OE-lines, grown in MS_0 and treated with the synthetic auxin, where the transcript level was 8-times fold increase, and in RNAi-lines – 4-fold increase to wit in WT ($p<0.01$; $p<0.01$). Lastly, *MtLAX3* relative transcript level was comparable in non-treated with 2,4-D leaves and roots, but after treatment root response was much stronger.

In the upper part of the plants, according to different content of Pi in the medium, in the transgenic lines and WT, it was established similar expression level of *MtMAX2*, before and after 2,4-D treatment (Figure 4). The lowest expression of the gene was detected in OE-lines grown in 2 mM Pi, and highest in the RNAi-lines grown in same Pi content and additionally treated with 2,4-D (2 mM Pi+2,4-D). In roots *MtMAX2* gene was expressed in different quantities, than in leaves (Figure 4). In roots and normal conditions the expression level of the investigated transcripts was significantly reduced in WT, compared to OE and RNAi-lines ($p<0.01$; $p<0.01$). After treatment with low and high Pi concentration *MtMAX2* expression profile was similar, with downregulation in OE and RNAi-lines compared to WT ($p=0.05$; $p<0.01$). After 2,4-D treatment significant up-regulation was observed in RNAi-lines previously grown in normal conditions, compare to WT and OE-lines ($p<0.01$; $p<0.01$). Slight increase of the transcript level was established when WT and RNAi grown in 1 µM+2,4-D, and in OE and RNAi-lines grown in 2 mM+2,4-D. In general, treatment with 2,4-D led to highest stimulation of the gene expression in RNAi-lines grown in normal conditions.

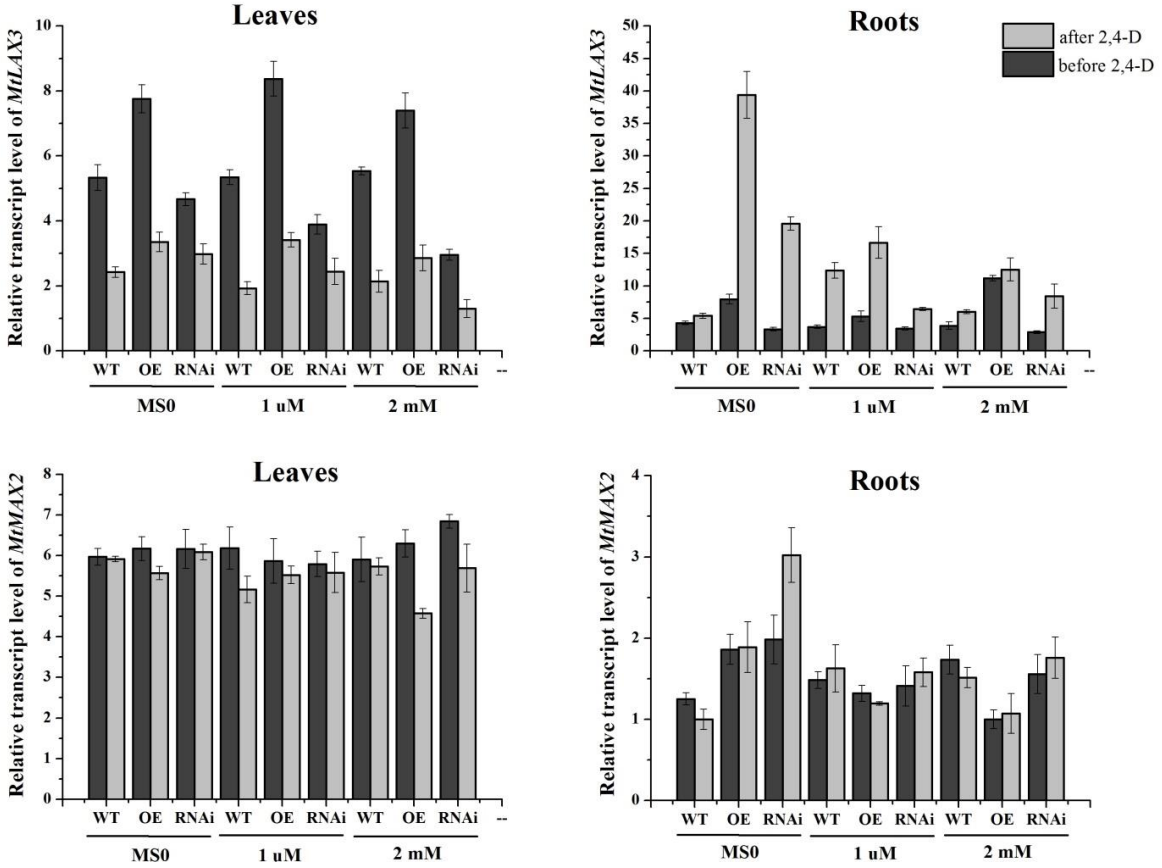

Figure 4. Relative expression level of *MtLAX3* and *MtMAX2* in leaves and roots of T_1 *M. truncatula* OE and RNAi lines, and WT plants grown in different phosphate conditions, before and after 2,4-D treatment.

3.3. Analysis of GUS activity in transcriptional reporter plants of M. truncatula, before and after 2,4-D treatment

Proven by selective PCR T_1 progeny of GUS positive transgenic plants of *M. truncatula* were used for histochemical analyses. *MtLAX3* expression pattern was followed during development of T_1 seedlings of *MtLAX3*-transcriptional reporter plants (*pMtLAX3::GUS-GFP*) of *M. truncatula*, germinated and grown on basal medium with different concentration of Pi - 1 µM, 2 mM P_i and basal MS_0 medium for 14 days (Figure 5) and additionally treated with 2,4-D for another 7 days (Figure 6). In normal MS_0 conditions the expression of GUS marker gene under the control of LAX3 promoter was followed in the vascular system of the leaves, main root and secondary branches, and in the internode of 14 dpg plants (Figure 5). Very light signal in the hypocotyls. In Pi-deprivation conditions strong GUS activity was reported in the vasculature of cotyledons, leaves and main root, and lateral buds and the internode. The observed blue colouring was more intensive in comparison with normal conditions and the roots of low-Pi-treated seedlings were brownish in colour compared to the others. The excess of Pi led to very light GUS expression in the vascular tissue of cotyledons and high expression in the vascular system of the leaves, main and lateral roots (Figure 5).

Further, the transcriptional reporter plants were treated with exogenous 2,4-D for another 7 days. *MtLAX3* expression observed was similar as not treated with 2,4-D plants (Figure 6) – in the vascular system of leaves, roots and lateral roots, and light signal in the cotyledons and internodes. GUS activity was detected in the root cap in plants grown in MS_0+2,4-D, where the signal was concentrated in the columella and stele. In low and high Pi supplemented with 2,4-D, *MtLAX3* signal was not detected in the root cap in the main root. In seedling grown at 2 mM Pi+2,4-D GUS signal was found in the root cap of secondary root branches (Figure 6).

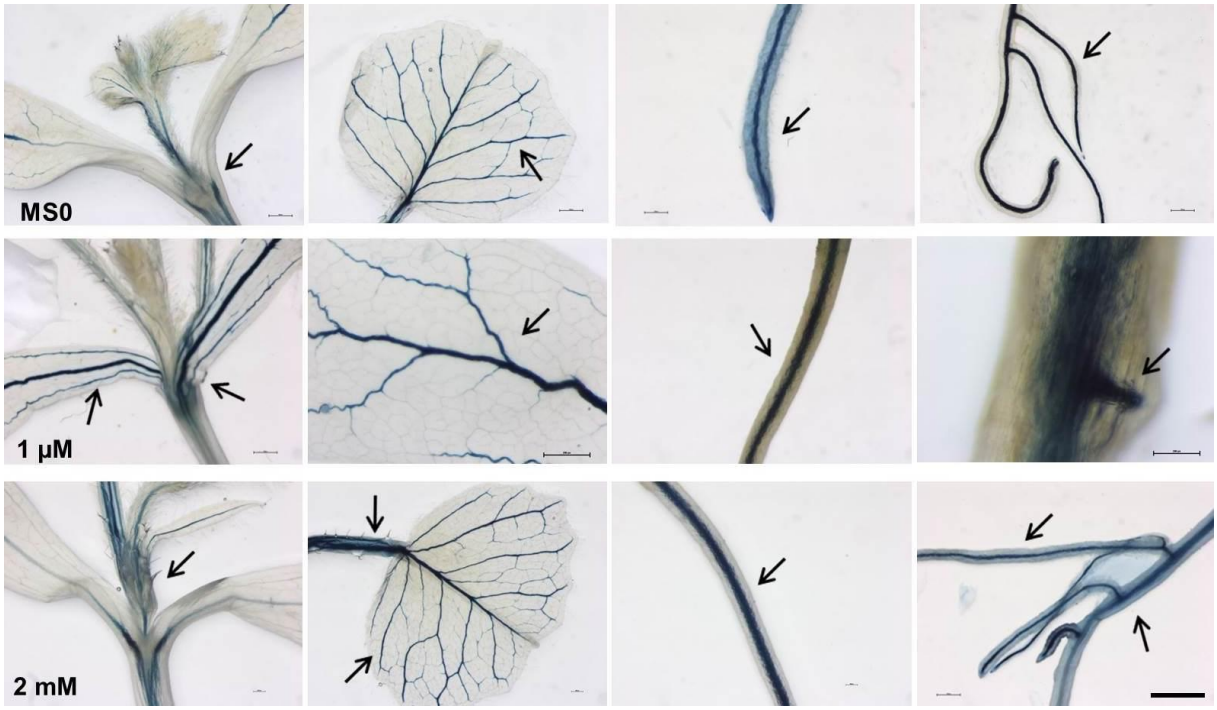

Figure 5. Expression pattern of *pMtLAX3::GUS::GFP* in T_1 *M. truncatula* lines grown in different phosphate conditions. The arrows pointed the expression of GUS reporter gene fused to the promoter of *MtLAX3* in the vascular system of cotyledons, leaves and roots, in lateral roots buds, and in internodes. Scale bar = from 0.5 to 3 cm.

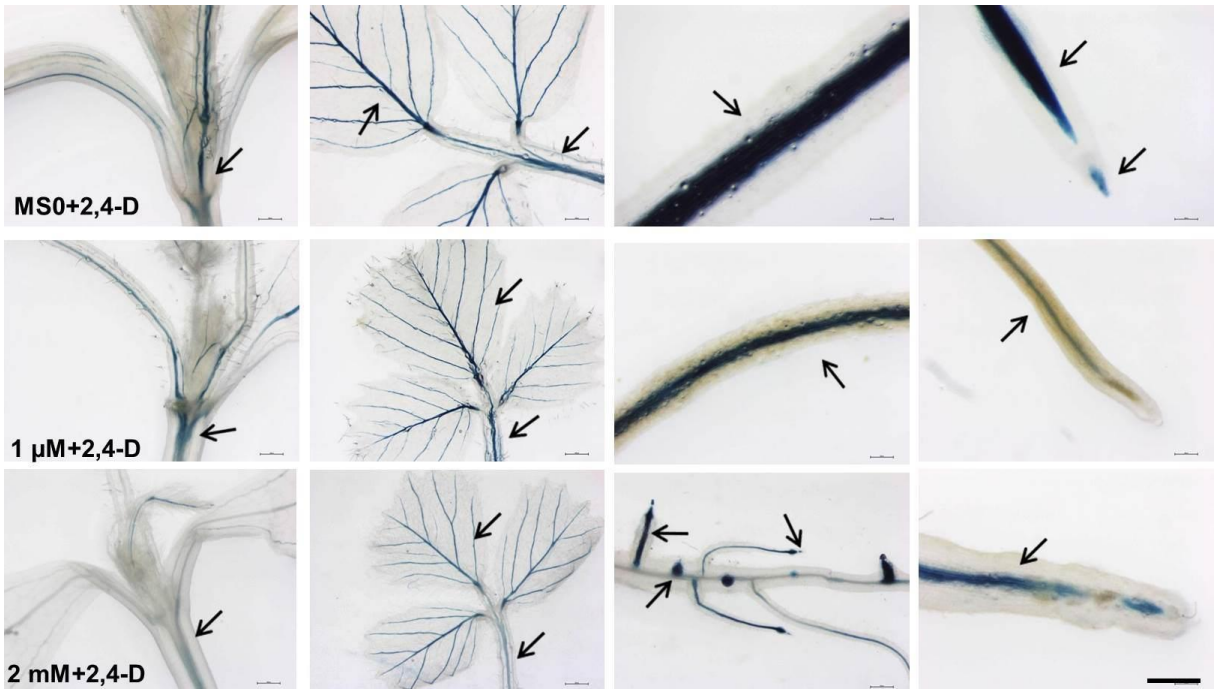

Figure 6. Expression pattern of *pMtLAX3::GUS::GFP* in T_1 *M. truncatula* lines grown in different phosphate conditions supplemented with 2,4-D. The arrows pointed the expression of GUS reporter gene fused to the promoter of *MtLAX3* in the vascular system of cotyledons, leaves and roots, root cap, in lateral roots buds, and in internodes. Scale bar = from 0.5 to 3 cm.

3.4. Statistical analysis

In this research we have examined the relationship between the two factors treatment and genotype and interaction effects. We have used Two-Way ANOVA is an appropriate method to analyse the main effects of and interactions between the investigated two factors. We wanted to determine the effects of treatment and genotype group on main root length (MRL), lateral root number (LRN) and relative expression level of two genes – *MtLAX3* and *MtMAX2* in leaves and roots. The "treatment group" factor includes six groups: normal conditions (MS_0); Pi-deprived conditions (1 µM) and Pi-excess conditions (2 mM); MS0+2,4-D; 1 µM+2,4-D; 2 mM+2,4-D. The "genotype group" includes three groups: OE-lines; RNAi-lines and 2HA control plants (WT). To find out of the treatment and genotype groups are independent or they interact with each other, we we have constructed a two-way ANOVA model predicting mean MRL, LRN and relative expression (*MtLAX3* and *MtMAX2*) level.

Two-way ANOVA analysis for MRL and LRN revealed significance between genotypes and treatment and the effect on the outcome of the change in "genotype" depend on the level of "treatment". To further detect interaction, we performed Tukey's test as the means comparison method. The results have shown the difference of the mean MS_0, 1 µM and 2 mM are significant at the level 0.05 for factor "treatment". The mean of OE, RNAi and WT are significant at the level 0.05 for factor "genotype".

The descriptive statistics of Two-Way ANOVA for relative expression level of the two investigated genes - *MtLAX3* and *MtMAX2* revealed that "treatment" and "genotype" are both significant factors, and the interaction between them it is significant for the expression level of *MtLAX3* and *MtMAX2* in roots, but in leaves the interaction is significant only for *MtLAX3* gene. Tukey's test was performed and revealed the mean of MS_0, 1 µM and 2 mM are significant at the level 0.05 for factor "treatment". The mean of OE, RNAi and WT are significant at the level 0.05 for factor "genotype".

4. DISCUSSION

The main purpose of this study was to understand what is the effect of exogenous application of 2,4-D on *M. truncatula* plants with modified auxin transport grown in extreme phosphate conditions. Therefore we have grown T_1 seeds from the progeny of transgenic *M. truncatula* plants with *MtLAX3* OE, downregulation and transcriptional reporters, as well as WT, on medium with different content of phosphate (KH_2PO_4): normal - 0.63 mM, low - 1 µM and high - 2 mM for 14 days. Afterwards the seedling were moved to the same media supplemented with 0,1 µM 2,4-D for additional 7-days treatment. For Pi is known to be the most easily absorbed by plants form of phosphorus, which is often the limiting factor for their normal growth and development (Bieleski 1973; Maathuis 2009). 2,4-D is synthetic auxin, mainly used as a herbicide, which is translocated through the leaves to the plant meristem (Cobb & Reade 2010; Simon & Petrasek 2011). Dellbare et al. (1996) have shown that 2,4-D is a substrate for LAX-transporters, where about 75% thereof requires "influx" carrier to enter the cell. In Revalska & Iantcheva (2018) we try to clarify the role and the interaction of the two plant hormones auxin and strigolactone in terms of phosphate starvation or excess. With this research we have expand the knowledge gained, by studying the exogenous effect of 2,4-D in terms of extreme phosphate conditions.

Three independent *M. truncatula* T_1 transgenic lines from OE, RNAi and transcriptional reporter constructs, and WT plants were monitored for phenotypic alterations and differences in the MRL and LRN, the expression level of *MtLAX3* and *MtMAX2*, before and after 2,4-D treatment. In Pi-deprivation medium MtLAX3-OE and RNAi-lines, and the WT possess shortened main root and increase of the LRN, compared to same lines in normal conditions. These results confirmed previous studies in *A. thaliana*, where in terms of Pi-deprivation the main root growth was inhibited (Niu et al. 2013; Giehl, Gruber & Wiren 2014), but formation of secondary roots was induced (Perez –Torres et al. 2008; Péret et al. 2014). One of the most typical structural changes of the root system, associated with a deficiency of phosphate, is variable root architecture (Péret et al. 2011, 2014; Mayzlish-Gati et al. 2012). OE and RNAi-lines, and WT plants treated with high concentration of Pi possess MRL and LRN comparable to the phenotype observed in plants grown in normal conditions. When 2,4-D was

added to the media, we observed drastic increase in the LRN in the transgenic and WT plants, but in MRL significant growth was not observed. It is known that the main root shortening was due to reduced elongation of the cells and in parallel reduces the population of stem cells in the primary root (Péret et al. 2014). On the other hand auxin is involved in the plant response to changes in the phosphate signaling. Also, changes in root architecture caused by phosphate deprivation are most often controlled by auxin signaling cascade. In our previous research we assumed that *MtLAX3* activates phosphate cascade by transporting auxin through the cell membrane into the cell (Revalska & Iantcheva 2018). We have allowed that phosphorus deficiency activates the function of *MtLAX3* and consequently stimulates secondary rooting and inhibition of main root growth. Also, plants grown under phosphorus deficiency are more sensitive to exogenous added auxin from plants grown with an excess of phosphorus, which resulted in the stunted growth of the main root and induced formation of secondary roots (Nacry et al. 2005; Chiou & Lin 2011; Lavenus et al. 2013). Low concentration of phosphorus stimulates secondary roots formation through increased sensitivity of roots to auxin (Péret et al. 2014). In addition, it is known about the auxin receptor TIR1 is required for the modulation of LR formation due to Pi availability (Perez –Torres et al. 2008). *LAX3* is responsible for auxin influx in the cells (Swarup et al. 2004, 2008), so in *M. truncatula* plants overexpressing *MtLAX3* auxin transport is more intensive due to increased expression of membrane transporters and the number of lateral roots was higher. In Revalska & Iantcheva (2018), we have assumed that in the present case *MtLAX3* activates phosphate cascade which stimulates secondary rooting. Secondary roots are very important for Pi-accumulation from the plants, since this vital mineral is located on the surface of the soil in normal environmental conditions, and by their lateral roots plants are capable to take over Pi for their needs.

In the present study additional treatment with 2,4-D led to extreme stimulation of lateral roots development in the transgenic and WT plants, previously grown at 1 µM, 2mM or MS_0 media, but the MRL was slightly increased. For 2,4-D is known to be substrate for LAX-transporters (Dellbare et al. 1996), and *MtLAX3* is involved in the polar auxin transport (Swarup et al. 2001), so the additional treatment with 2,4-D led to stimulation of the lateral roots development. In normal conditions LRN in OE-lines was many times higher than in the low or high Pi-conditions, where the plants were suffering from phosphate deprivation or excess. Nevertheless in extreme Pi-conditions 2,4-D acts as an activator of the *MtLAX3* and respectively activates lateral root development. In the RNAi-lines the polar auxin transport is disturbed and most likely due to the exogenous application of 2,4-D in the media, most of it enters the cell through diffusion and stimulates lateral root growth. The combination of high concentration of Pi in the medium and the additional 2,4-D treatment led to increased LRN in comparison with normal conditions. So, we could propose that high Pi-content in media and more 2,4-D could compensate the disturbed auxin transport and safe the phenotype. The observed transgenic lines together with WT possess darkening in the root system when grown on Pi-deprivation medium. Obviously, the root cells became brownish due to the low Pi concentration. In plants phosphorus is a mobile and is translocate among the plant, causing necrosis and chlorosis (Sanchez 2015).

The results from qRT-PCR analysis have shown differences in the expression of *MtLAX3* and *MtMAX2* in the different plant parts – leaves and roots, before and after 2,4-D treatment. In leaves *MtLAX3* expression in OE-lines grown in low concentrations of Pi was higher in comparison with normal and high conditions. For the OE, RNAi-lines and WT the expression profile was the same in the 3 different media – *MtLAX3* upregulation for the OE and downregulation for the RNAi, to wit WT. After 2,4-D treatment the expression level of *MtLAX3* was downregulated, probably due to the fact that auxin is mainly synthesized in the young leaves and apical meristem of the shoot and roots (Chen et al. 2014), but by a combination of carrier-mediated uptake and diffusion is transported acropetally from the shoot to the root (Friml et al. 2003; Teale, Paponov & Palme 2006). The level of *MtMAX2* expression in leaves was almost the same in the different media and in different constructs, and WT. These results have shown that in leaves different Pi-content did not affect the expression of the strigolactone gene, but treatment with 2,4-D slightly reduced the transcript level.

In roots high Pi content in the media led to upregulation of *MtLAX3* gene for 14 days treatment compare to normal and low Pi conditions. Due to the polar auxin transport, after 2,4-D treatment *MtLAX3* transcript level was up to 4-fold upregulated in comparison to non-treated lines. This was

obvious from the obtained phenotype – drastic increase in the LRN. The expression level of *MtMAX2* in roots was highest in normal conditions in the transgenes and WT, compare to the extreme Pi-content. The expression response after 2,4-D treatment was not one-sided – upregulation in the RNAi-lines grown in the 3 different media, and slight downregulation or very low upregulation in the OE and WT. Pi availability modulates the expression of the TIR1 auxin receptor in *Arabidopsis* seedlings (Perez–Torres et al. 2008) and under conditions of Pi depletion the strigolactone pathway, through *MAX2* activates TIR1 expression and the auxin signaling pathway, and thus regulate root development to suit those growth conditions (Mayzlish-Gati et al. 2012). Also, strigolactones may regulate the local levels of auxin in plants, either by changing the biosynthesis of auxin or polar auxin transport (Rasmussen et al. 2013; Shinohara et al. 2013). Therefore we may assume *MtLAX3* gene acts as activator of the phosphate cascade (Revalska & Iantcheva 2018).The exogenous application of 2,4-D activates the cascade of auxin and strigolactone signaling, which leads to the upregulation in *MtLAX3* and *MtMAX2* even in RNAi-lines, where the auxin transport was disrupted.

*MtLAX3*expression pattern monitored by GUS activity assay was further investigated. *MtLAX3* expression was observed in the vascular system of the leaves, cotyledons, and roots, root cap and in internodes. 14 dpg, the GUS signal was enhanced in the seedlings grown in Pi-deprivation medium in comparison with normal conditions, while treatment with high concentration of Pi was comparable with normal conditions. The root cells of seedlings treated with low Pi became darkened due to necrosis caused by Pi deficiency. Same symptom was established in the transgenic and WT plants grown in Pi-deficiency. These results confirmed the results obtained in Revalska & Iantcheva (2018) for 21 dpg transcriptional reporter seedlings. After 2,4-D treatment, we monitored differences in the expression pattern of *MtLAX3*, where GUS signal was not observed in the root cap of the main root, but it was strong in the root cap of lateral roots.

5. CONCLUSIONS

As a general conclusion we could state drastic changes in the root architecture were observed in the transgenic and WT plants, placed in extreme conditions of phosphate deficiency and excess. After the performed phenotypic and biometric analysis we can conclude that low Pi-concentration in the medium led to significant reduction in MRL for all of the investigated constructs and WT for 14 days treatment. The high Pi content led to not significant reduction in the MRL for the same time point. Pi-starvation and Pi-excess did not reflect the LRN in OE, RNAi-lines and WT in comparison with normal conditions. The additional 7-days treatment with 2,4-D led to dramatic stimulation of the LRN in the transgenic and WT plants, where 2 mM Pi in combination with the auxin could save the phenotype for the RNAi-lines.

All of the transgenic lines and WT were characterized by high plasticity of surviving phosphorus starvation or excess, after exogenous application of 2,4-D, due to the significant increase in LRN and the relative expression level of *MtLAX3* and *MtMAX2* in roots. 2,4-D activates the function of *MtLAX3* and consequently activates the *MtMAX2* and stimulates secondary rooting and inhibition of main root growth. The obtained results strongly contributed to understanding the interaction between auxin and strigolactones, their function in plant growth and development and can be used to optimize the crop growth of legumes in eroded and poor of phosphate soils.

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