Türk Tarım ve Doğa Bilimleri Dergisi 7(4): 1043–1048, 2020

https://doi.org/10.30910/turkjans.782572

TÜRK
TARIM ve DOĞA BİLİMLERİ
DERGİSİ

TTDB

TURKISH
JOURNAL of AGRICULTURAL
and NATURAL SCIENCES

www.dergipark.gov.tr/turkjans

Research Article

The Role of miRNA408 in Phosphate Deficiency Stress of *Lolium perenne*: A Three-Year Field Experiment

Gürkan DEMİRKOL

Ordu University, Faculty of Agriculture, Department of Field Crops, Ordu
Corresponding author: gurkandemirkol@odu.edu.tr

Received: 19.08.2020 Revised in received: 05.10.2020 Accepted: 14.10.2020

Abstract

Phosphate deficiency is a stress factor limiting crop growth and development. Under phosphate limited conditions, crops have developed a variety of molecular strategies. miRNAs (miRs) are characterized as a regulator of main processes such as stress mechanisms in plants by silencing genes. miR408 has role in mediating plant responses to phosphate starvation in limited number of plants. The miRs involved in the phosphate deficiency mechanism in *Lolium perenne* (perennial ryegrass) remain unelucidated. The aim of this study was to confirm the presence of miR408 expression in perennial ryegrass and control whether it plays an important role to protect against phosphate deficiency stress in field conditions. The sensitivities (hay yield and quality features) of four perennial ryegrass populations against phosphate deficiency stress were determined by three-year field experiment. The results revealed that the declines on hay yield and quality features were less pronounced for P1 and P4 populations compared to others (P2, and P3) against phosphate deficiency stress. Molecular analysis showed that significant up-regulations were observed in the expression level of the miR408 in P1 and P4, while no changes were observed in P2, and P3. The results collectively suggest that miR408 could be responsible for the tolerance in phosphate-limited conditions in perennial ryegrass. This miR could be used for the development of perennial ryegrass plants that are tolerant to phosphate-deficient soils.

Key words: Abiotic stress, field study, forage crop, phosphate starvation

Lolium perenne'de Fosfat Eksikliği Stresinde miRNA408'in Rolü: Üç Yıllık Tarla Çalışması

Öz

Fosfat eksikliği, bitkilerde büyüme ve gelişmeyi sınırlandıran bir stres faktörüdür. Fosfat eksikliği koşullarında bitkiler birtakım moleküler stratejiler geliştirmiştir. miRNA (miR)'lar, genleri susturarak bitkilerdeki stres mekanizmaları gibi ana süreçlerin düzenleyicisi olarak karakterize edilirler. miR408, sınırlı sayıda bitkide fosfat eksikliğine karşı role sahiptir. Bugüne kadar *Lolium perenne* (çok yıllık çim) bitkisinde fosfat eksikliği mekanizmasında yer alan miR'ler yeterince açıklanmamıştır. Bu çalışmanın amacı, miR408 ekspresyonunun çok yıllık çim bitkisinde varlığını doğrulamak ve tarla koşullarında fosfat eksikliği stresine karşı önemli bir rol oynayıp oynamadığını belirlemektir. Çalışmada altı adet çok yıllık çim populasyonunun fosfat eksikliği stresine karşı duyarlılıkları (kuru ot verimi ve kalite özellikleri) üç yıllık tarla çalışması ile belirlenmiştir. Tarla çalışmalarından elde edilen sonuçlar kuru ot verimi ve kalite özelliklerindeki düşüşlerin, fosfat eksikliği stresine karşı diğerlerine (P2 ve P3,) kıyasla P1 ve P4 populasyonları için daha az olduğunu ortaya koymuştur. Moleküler analiz sonuçları dikkate alındığında, fosfat eksikliği stresi altında P1 ve P4 populasyonlarında miR408'in ekspresyon seviyesinde önemli artış gözlemlenirken, P2 ve P3'te herhangi bir değişiklik tespit edilmemiştir. Elde edilen sonuç miR408'in çok yıllık çim bitkisinde fosfat eksikliği stresine toleranstan sorumlu olabileceğini göstermektedir. Bu miR, fosfat eksikliğine karşı dayanıklı çok yıllık çim bitkisi geliştirme amaçlı kullanım potansiyeline sahiptir.

Anahtar kelimeler: Abiotik stres, tarla çalışması, yem bitkisi, fosfat eksikliği

Introduction

Phosphorous, an essential macronutrient for plant growth, development, and reproduction. Despite the importance of phosphorous in agricultural production, most phosphorous in the soil is unavailable for plant because of the insufficient availability of soluble phosphate (Pei et al., 2013). Phosphate deficiency causes death or decreases in yield and quality in plants. In response to phosphate deficiency, plants have evolved a number of strategies, which provide many processes to enhance phosphate utilization.

miRs are known as regulators of many processes in plants against stress factors. Recent studies show that miRs can be involved with phosphate deficiency in several plant species (Zeng et al., 2016; Li et al., 2018; Ning et al., 2019). miR408 is one of the highly conserved miRNA family member in several plant species (Hajyzadeh et al., 2015). Previous studies have indicated that the transcriptional response of miR408 against several abiotic stress factors such as drought and phosphate starvation (Hajyzadeh et al., 2015; Liang et al., 2015; Bai et al., 2018).

Perennial ryegrass is the most widely used forage crop in temperate regions due to its high nutritional value (Huang et al., 2014). To date, the molecular mechanism involved in the phosphate deficiency mechanism in perennial ryegrass remains largely unknown.

The aim of this study was to confirm the presence of miR408 expression in perennial ryegrass and control whether it plays an important role to mediate phosphate deficiency stress in field conditions.

Materials and Methods

Plant materials

Four populations (P1, P2, P3, and P4) were used that were collected from the natural flora of Turkey.

Experimental setup growth conditions

A field study was conducted in randomized complete block design with four replications in Ordu province/Turkey (40° 58' N, 37º 56' E, 10 m above sea level) during the years of 2016, 2017, and 2018. The experiment was designed under normal or phosphate-deficient conditions.

The climate conditions was typical coastal area of Black Sea region climate characteristics (with a mean annual temperature of 15.6°C and a mean annual precipitation of 1146 mm. The values are 14.4°C and 1040 mm in long term of 1961-2019). The climatic values of the experiment area are listed in Table 1.

Table 1. Climatic values of the experiment area.

Months	Mean temperature (°C)					Total precipitation (mm)				
	2015	2016	2017	2018	LT	2015	2016	2017	2018	LT
January	-	7.0	6.1	8.4	6.8	-	222.2	97.0	181.4	99.8
February	-	10.6	6.9	9.7	7.0	-	108.2	56.6	59.2	80.5
March	-	10.6	9.3	11.6	8.2	-	121.0	89.4	116.1	81.0
April	-	14.1	10.5	12.5	11.4	-	39.9	54.3	36.4	68.1
May	-	16.7	15.4	18.5	15.6	-	115.1	72.6	62.0	55.6
June	-	22.1	20.8	22.6	20.3	-	55.1	54.7	37.4	73.1
July	-	24.1	24.0	25.0	23.0	-	138.8	10.8	109.0	63.8
August	-	25.7	25.3	-	23.4	-	57.0	38.8	-	66.3
September	-	20.9	22.3	-	20.2	-	158.6	29.6	-	81.2
October	17.3	16.2	16.4	-	16.1	241.7	99.4	85.0	-	131.7
November	14.1	12.1	13.0	-	12.1	74.3	127.9	63.0	-	123.2
December	8.5	6.3	11.2	-	8.9	156.7	190.6	137.8	-	116.5

*LT: Long Term (1961-2019)

** The data were taken from Ordu Meteorology Directorate (https://mgm.gov.tr/)

The soil of experimental area was a clay-loam, neutral (pH: 6.89), salt-free (EC: 470 µS cm-1), moderate in organic matter (2.52%), adequate in terms of nitrogen (0.118 %) inadequate in phosphorus (6.2 mg/kg) and adequate potassium (64 kg/da).

The treatments consisted of control (0 kg/ha P2O5), and fertilized (60 kg/ha P2O5 before seeding and in each following year). All treatments had the same N fertilizer level of 30 kg N/ha applied. The seeds were sown at seeding rates of 1 kg/da in 29th October 2015.

Each experimental unit (6 m2) included six rows, each 5 m in length, with a space of 0.20 m between rows and a space of 0.05 m between two consecutive plants in the same row.

The plants were harvested from a 4 m2 area of each unit when they were at 50% bloom stage.

In 2017, there were two harvests, and in subsequent years (2018 and 2019), the plots were cut three times per year. The samples from each plot was oven dried at 60 °C to enable determination of hay yield. In addition, the crude protein ratio, ADF (acid detergent fiber) and NDF (neutral detergent fiber) were determined with near-infrared reflectance spectroscopy (NIRS).

RNA isolation and real time (RT) PCR

RNAs were extracted using the method of Chomczynski and Sacchi (2006) with minor modifications. The RNA was used to synthesize cDNA using miR specific primer (AUGCACUGCCUCUUCCCUGGC) with Superscript reverse transcriptase III (Invitrogen) by following the manufacturer's instructions. A RT reaction protocol was; 30 min at 16ºC, 60 cycles at 30ºC for 30 s, 42ºC for 30 s and 50ºC for 1 s. 18S rRNA was used as an endogenous control.

Statistical analysis

The analyses were carried out in triplicate. SPSS 22 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Tukey's test was performed at the α=0.05.

Results and Discussion

Yield and quality parameters

The three-year field study results revealed that stress conditions caused significantly more severe declines in hay yield and crude protein ratio, whereas more increases in ADF and NDF in P2 and P3, compared to P1, and P4.

P2, and P3 showed dramatic reductions in hay yield under phosphate stress conditions (Figure 1). In the third year of the study, the hay yield of P2 and P3 decreased approximately three times compared to the control under phosphate deficiency stress. Studies have shown that phosphate deficiency results in decreases in photosynthesis, reduced shoot, leaf, and biomass (Li et al., 2018) which is consistent with the results of this study. Moreover, significant decreases were observed in third year compared to the previous years in P2, and P3 in hay yield. (Figure 1). Similar results were reported as the low phosphate availability in soils limits yield in most of the crops (Gupta et al., 2017; Venkatachalam et al., 2009). The findings clearly indicate that P1 and P4 have a potential in tolerance to phosphate deficiency in forage yield.

P2, and P3 showed decreases in crude protein each year of the study under stress condition, compared to normal condition (Figure 2). Moreover, these populations showed the lowest crude protein content in the third year of the study. Suprisingly, P1 and P4 maintained crude protein rates under stress condition.

Similar results were obtained in ADF and NDF rates (Figure 3, 4). While no change was observed in ADF and NDF in P1 and P4 (Figure 3, 4), P2, and P3 showed increases under stress condition. Increased ADF rates decreases the forage quality due to the increase of the non-digestible fiber content (Demirkol and Yilmaz, 2019). Similar decreases in crude protein ratio and forage quality under phosphate limited conditions were reported in phosphate-sensitive narbon vetch (Turk et al., 2007).

The three-year field study findings collectively showed that P1 and P4 are less sensitive to phosphate starvation than P2, and P3.

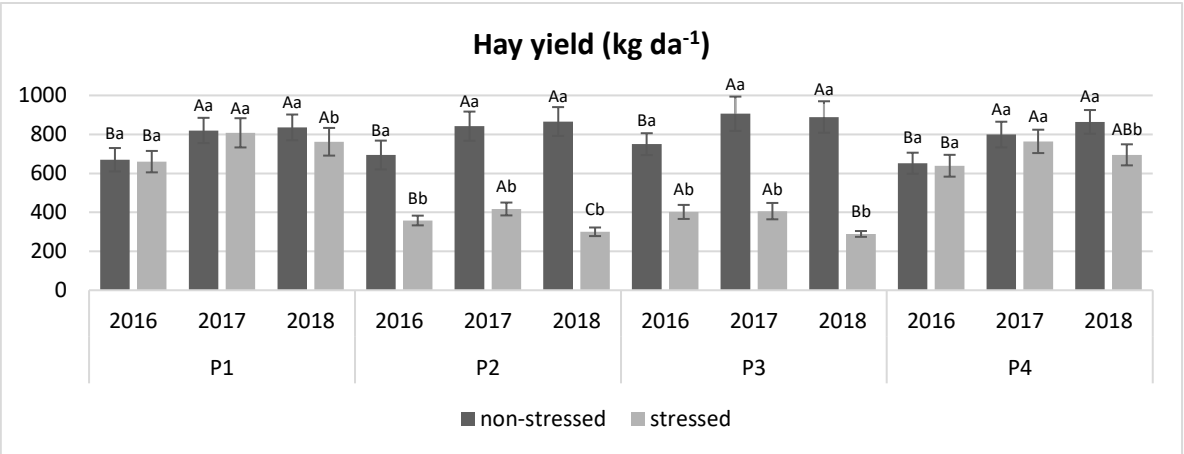

Figure 1. Hay yield (kg/da) of the populations under non-stressed and stressed conditions in three-year field study. Data represent mean±SD of triplicates. Values with the different capital letter in a population in a treatment indicate a significant difference between years at $p < 0.05$. Values with the different small letter in a population in a year indicate a significant difference between non-stressed and stressed populations at $p<0.05$.

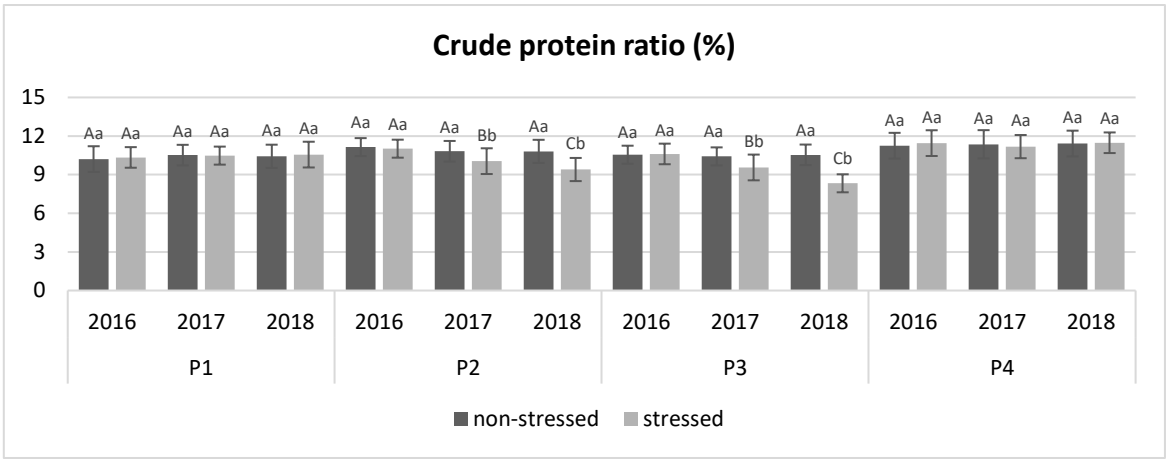

Figure 2. Crude protein ratio (%) of the populations under non-stressed and stressed conditions in three-year field study. Data represent mean±SD of triplicates. Values with the different capital letter in a population in a treatment indicate a significant difference between years at $p < 0.05$. Values with the different small letter in a population in a year indicate a significant difference between non-stressed and stressed populations at $p<0.05$.

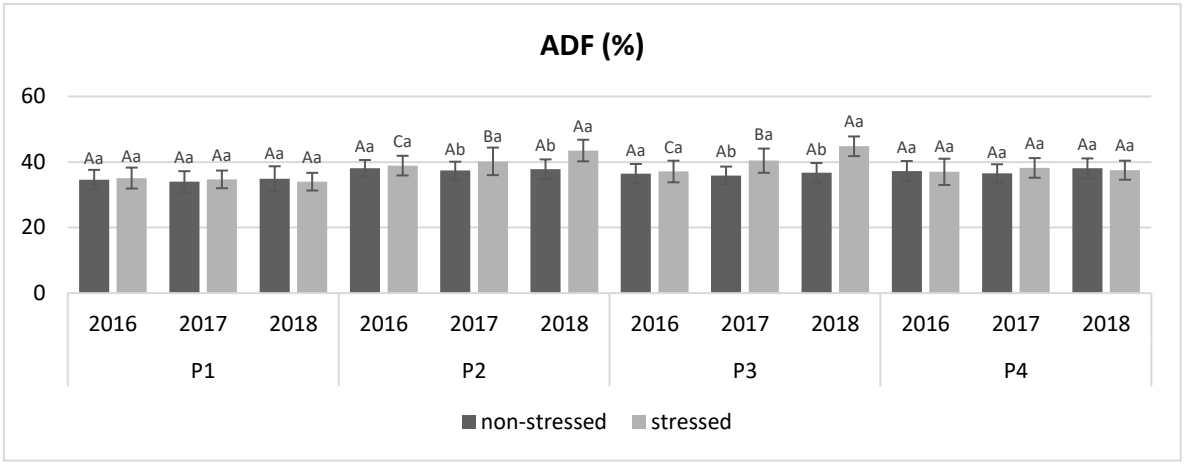

Figure 3. ADF rates (%) of the populations under non-stressed and stressed conditions in three-year field study. Data represent mean±SD of triplicates. Values with the different capital letter in a population in a treatment indicate a significant difference between years at $p < 0.05$. Values with the different small letter in a population in a year indicate a significant difference between non-stressed and stressed populations at $p<0.05$.

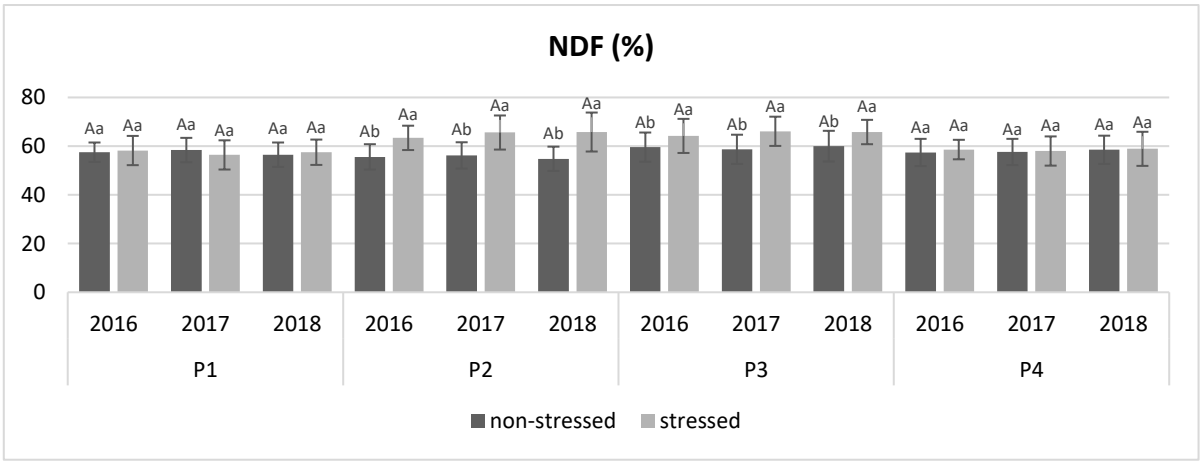

Figure 4. NDF rates (%) of the populations under non-stressed and stressed conditions in three-year field study. Data represent mean±SD of triplicates. Values with the different capital letter in a population in a treatment indicate a significant difference between years at $p < 0.05$. Values with the different small letter in a population in a year indicate a significant difference between non-stressed and stressed populations at $p<0.05$.

miRNA expression

The phosphate deficiency stress up-regulated the levels of miR408 in P1, and P4, while no change was observed in P2, and P3 (Figure 5). On the other hand, considering the difference between years, the highest expression levels were observed in the third year in P1, and P4. The results indicate that miR408 could be acceptable as responsible for phosphate-stress tolerance in perennial ryegrass.

Increasing evidences suggest that miR408 is crucial for plant adaptations to phosphate starvation through regulating phosphate acquisition (Bai et al., 2018; Pei et al., 2013). The findings of this study indicated the possible connection between miR408 and phosphotransferase genes which mediate phosphate deficiency under phosphate-limited conditions. Plant phosphotransferase genes share conserved function in promoting phosphate uptake from media into root cells and regulating internal phosphate translocation (Bai et al., 2018; Shin et al., 2004).

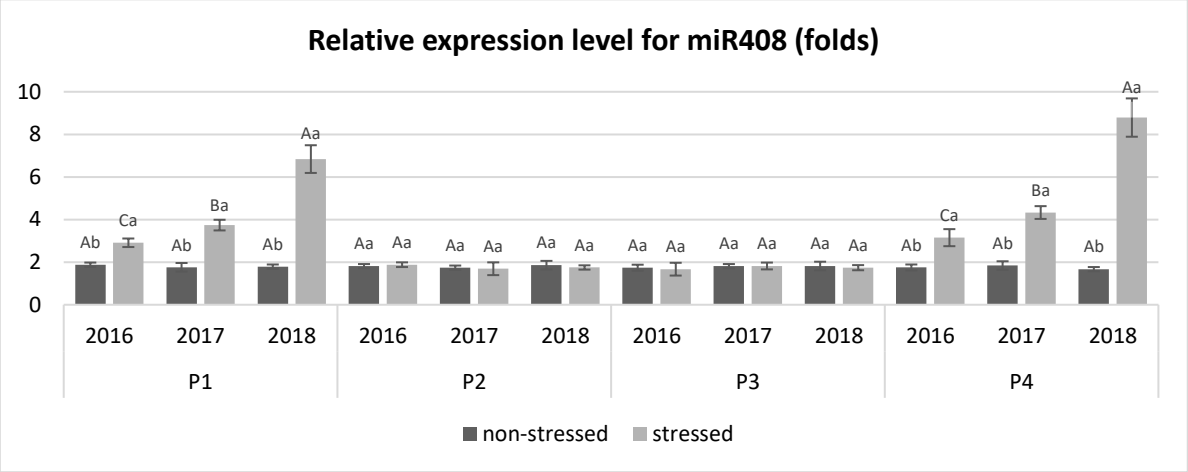

Figure 5. Relative expression level for miR408 of the populations under non-stressed and stressed conditions in three-year field study. Data represent mean±SD of triplicates. Values with the different capital letter in a population in a treatment indicate a significant difference between years at $p < 0.05$. Values with the different small letter in a population in a year indicate a significant difference between non-stressed and stressed populations at $p<0.05$.

Conclusion

As a result of the study, it was observed that phosphate deficiency caused dramatic yield and quality losses in P2, and P3, while P1, and P4 were less affected. Molecular analysis showed that while the expression levels of the miR408 did not changed in P2, and P3, significant up-regulations were observed in P1 and P4. The results of this study have advanced our understanding of miRs-mediated gene regulation in perennial ryegrass that could be potential targets for agricultural productivity which require less phosphate fertilization. New perennial ryegrass having up-regulated miR408 could be used to develop that tolerate phosphate deficiency.

Conflict of Interest Statement: The manuscript's authors declare that, they do not have any conflict of interest.

Researchers' Contribution Rate Statement Summary: The authors declare that, they have contributed equally to the manuscript.

References

Bai, Q., Wang, X., Chen, X., Shi, G., Liu, Z., Guo, C. and Xiao, K. 2018. Wheat miRNA TaemiR408 acts as an essential mediator in plant tolerance to Pi deprivation and salt stress via modulating stress-associated physiological processes. *Frontiers in Plant Science*, 9: 1-17.

Demirkol, G. and Yilmaz, N. 2019. Forage pea (*Pisum sativum* var. *arvense* L.) landraces reveal morphological and genetic diversities. *Turkish Journal of Botany*, 43 (3): 331-342.

Gupta, S., Kumari, M., Kumar, H. and Varadwaj, P. K. 2017. Genome-wide analysis of miRNAs and Tasi-RNAs in *Zea mays* in response to phosphate deficiency. *Functional & Integrative Genomics*, 17 (2-3): 335-351.

Hajyzadeh, M., Turktas, M., Khawar, K. M. and Unver, T. 2015. miR408 overexpression

causes increased drought tolerance in chickpea. *Gene*, 555 (2): 186-193.

Huang, Y., Zou, Q., Sun, X. H. and Zhao, L. P. 2014. Computational identification of microRNAs and their targets in perennial ryegrass (*Lolium perenne*). *Applied Biochemistry and Biotechnology*, 173 (4): 1011-1022.

Li, Z., Xu, H., Li, Y., Wan, X., Ma, Z., Cao, J., Li, Z., He, F., Wang, Y. and Wan, L. 2018. Analysis of physiological and miRNA responses to Pi deficiency in alfalfa (*Medicago sativa* L.). *Plant Molecular Biology*, 96 (4-5): 473-492.

Liang, G., Ai, Q. and Yu, D. 2015. Uncovering miRNAs involved in crosstalk between nutrient deficiencies in Arabidopsis. *Scientific Reports*, 5: 1-13.

Ning, L., Du, W., Song, H., Shao, H., Qi, W., Sheteiwy, M. S. A. and Yu, D. 2019. Identification of responsive miRNAs involved in combination stresses of phosphate starvation and salt stress in soybean root. *Environmental and Experimental Botany*, 167, 1-16.

Pei, L., Jin, Z., Li, K., Yin, H., Wang, J. and Yang, A. 2013. Identification and comparative analysis of low phosphate tolerance-associated microRNAs in two maize genotypes. *Plant Physiology and Biochemistry*, 70: 221-234.

Shin, H., Shin, H. S., Dewbre, G. R. and Harrison, M. J. 2004. Phosphate transport in Arabidopsis: Pht1; 1 and Pht1; 4 play a major role in phosphate acquisition from both low-and high-phosphate environments. *The Plant Journal*, 39 (4): 629-642.

Turk, M., Albayrak, S. and Yüksel, O. 2007. Effects of phosphorus fertilisation and harvesting stages on forage yield and quality of narbon vetch. *New Zealand Journal of Agricultural Research*, 50 (4): 457-462.

Venkatachalam, P., Jain, A., Sahi, S. and Raghothama, K. 2009. Molecular cloning and characterization of phosphate (Pi) responsive genes in Gulf ryegrass (*Lolium multiflorum* L.): a Pi hyperaccumulator. *Plant Molecular Biology*, 69 (1-2): 1-21.

Zeng, H., Wang, G., Zhang, Y., Hu, X., Pi, E., Zhu, Y., Wang, H. and Du, L. 2016. Genome-wide identification of phosphate-deficiency-responsive genes in soybean roots by high-throughput sequencing. *Plant and Soil*, 398 (1-2): 207-227.