YEM BEZELYESİNİN FOSFİNOTRİSİN TOLERANSININ BELİRLENMESİ

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Özet

Bu çalışma, bir selektif markör olan fosfinotrisinin yem bezelyesine gen transferi uygulamasındaki en uygun dozunu belirlemek için yapılmıştır. Fosfinotrisinler, *Streptomyces* toprak bakterisi tarafından üretilen geniş spektruma sahip bir herbisitlerdir. Bu herbisitlerin bitkilere uygulanması ile yapılarındaki glutamin sentetazın inhibisyonu glutaminin azalmasına ve amonyak miktarının artmasına neden olarak fotosentezi durdurmakta ve bitkinin ölümüne sebep olmaktadır. Çalışma, Sakarya Uygulamalı Bilimler Üniversitesi Ziraat Fakültesi Tarla Bitkileri laboratuvarında gerçekleştirilmiştir. Bu amaçla in vitro ortamda kontrol, 2, 4 ve 6 mg/L fosfinotrisin içeren Murashige-Skoog (MS) besin ortamı kullanılmıştır. Uygulanan dozlar sonucunda çimlenme oranı, kök ve sürgün uzunluğu, kök ve sürgün ağırlıkları incelenmiştir. Deneme 3 tekerrürlü olarak tesadüfi parselleri deneme desenine göre yürütülmüştür. Elde edilen sonuçlar, kontrol grubuna göre çimlenme oranı, kök ve sürgün uzunluğu, kök ve sürgün ağırlıkları azalış göstermiştir. Çimlenme oranı sırasıyla %100, %90, %70 ve %20 olarak bulunmuştur. Kontrol grubuna göre 2 mg/L fosfinotrisin miktarındaki sürgün uzunluğunda %50 azalma gözlenmiştir. Çalışmada kotiledon ve kök boğum olmak üzere 2 eksplantlar kullanılmıştır. Bunun sonucunda ise hiçbir dozda kök ve sürgün oluşumu gelişme gözlenmemiştir. Sonuç olarak yem bezelyesinde gen transferi çalışmaları için kritik fosfinotrisin miktarı 2 mg/L olarak bulunmuştur.

Anahtar Kelimeler: Yem bezelyesi (*Pisum arvense L.*), fosfinotrisin, çimlenme, fide gelişimi.

DETERMINATION OF FORAGE PEA TOLERANCE TO PHOSPHINOTHRICIN

Abstract

The present study was conducted to determine the optimal dose of phosphinothricin, a selective marker, for gene transfer application to forage peas. Phosphinothricins are broad-spectrum herbicides produced by *Streptomyces* soil bacteria. When applied to plants, these herbicides inhibit glutamine synthetase in their structure, reducing glutamine and increasing ammonia, which stops photosynthesis and causes plant death. The study was carried out at Sakarya University of Applied Sciences, Faculty of Agriculture, Field Crops laboratory. For this purpose, control, Murashige-Skoog (MS) nutrient medium containing 2, 4 and 6 mg/L phosphinothricin were used in vitro. As a result of the doses applied, germination rate, root and shoot length, root and shoot weights were analyzed. The experiment was conducted using a randomised plot design with three replications. The results showed a decrease in germination rate, root and shoot length, root and shoot weight compared to the control group. Germination rates were 100%, 90%, 70% and 20%, respectively. A 50% decrease was observed in shoot length at 2 mg/L phosphinothricin compared to the control group. In the study, two explants, cotyledon and root-knot, were used. The results showed that there was no improvement in root and shoot formation at any dose. Consequently, the critical level of phosphinothricin for gene transfer studies in forage peas was found to be 2 mg/L.

Keywords: Forage pea (*Pisum arvense* L.), phosphinothricin, germination, seedling growth.

1. INTRODUCTION

With the start of agricultural production, weeds observed in agricultural areas are a significant problem and cause a loss of 26%-40% in agricultural production worldwide (Erdoğan, 2024). Herbicides with a molecular weight of less than 500 MW, used for weed control, are efficiently used during mechanical control to control weeds with similar morphology to the agricultural product (Ivanov, 2019; Kantwa vd., 2019).

The Herbicide Resistance Action Committee (HRAC) has classified herbicides into selective and non-selective herbicides. The selective herbicides, 2,4-D, dicamba and mecoprop, suppress the growth of target plants and do not affect the desired crop. Phosphinothricins, a group of non-selective herbicides, affect the entire plant when applied to the plants to which they are specific. Phosphinothricins are also known as glufosinates (Hussain vd., 2021).

1.1. Phosphinothricins

Phosphinothricins are a broad-spectrum herbicide produced by the soil bacterium *Streptomyces*. Phosphinothricins, a glutamic acid analog, are a residue of bialaphos, a natural antibiotic with a tripeptide structure produced by the bacteria *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus*. (Cui vd., 2016). Application of this herbicide to plants causes inhibition of glutamine synthetase in their structure, causing a decrease in glutamine and an increase in the amount of ammonia, stopping photosynthesis and causing plant death (Dmitrovic vd., 2021).

1.2. Forage pea (*Pisum arvense* L.)

Peas are one of the most widely cultivated legumes worldwide and are grown throughout the temperate zone. In EU countries, peas represent the most important legume crop. It is mainly grown for the seeds, which have a high protein content (21-24% crude protein), about twice as high compared to cereals. However, pea protein (similar to other legumes) contains low levels of essential, sulfur-containing amino acids.

Safe for animal health, reducing antinutritional substances such as trypsin inhibitors and phytic acid, which limit the digestibility of proteins and some minerals, especially phosphorus (Ludvíková and Griga, 2022). Forage pea, which has many superior properties, has been one of the most studied species by forage plant breeders in recent years. As a result of these intensive breeding studies, many new fodder pea varieties have been developed and offered to the service of producers (Sayar, 2021). The classical breeding process is quite

long and laborious, and the standard time to release a new pea variety is about 10 to 15 years (Ludvíková and Griga, 2022). An alternative to these breeding efforts is the application of recombinant DNA technology in agriculture. The aim of this technology is to impart a new trait to the plant by gene transfer.

In this research, the production of phosphinothricin (PPT)-resistant transgenic Pisum sativum L. plants will be achieved using Agrobacterium tumefaciens-mediated gene transfer. The protocol is based on the regeneration of shoots from cotyledon and root knot explants. Before inoculation with strain EHA105 harboring phosphinothricin resistance (bar) genes conferring resistance to PPT, it is necessary to determine the doses of selective antibiotics or herbicides used in the selection of gene-transferred shoots. Since the binary plasmids used in this study contain the bar gene and the bar gene makes the transferred plant cells resistant to PPT, it is of great importance to determine the amount of PPT to be used in selection media. For this purpose, 2, 4 and 6 mg/L phosphinothricin media were tested.

2. MATERIAL AND METHOD

2.1. Material

Ates forage pea obtained from Namık Kemal University Faculty of Agriculture was used as plant material in the study.

2.2. Method

This research was carried out at Sakarya University of Applied Sciences, Department of Horticulture, Plant Tissue Culture and Field Crops Laboratory and the seed sterilization and tissue culture optimization method used by Das et al. (2014) was adapted. In the study, 5% Start Industrial Cleaning Product, MS (Murashige and Skoog) (PhytoTech LAB, Catalog No. M404) media and three different (2, 4, 6 mg/L) phosphinothricin media were used for seed sterilization. The study was conducted in 3 replicates. MS 4.4, glucose 30 and agar 6 g were used in the study and after adjusting the pH 5.8, the sterilization process was carried out at 121°C for 20 minutes under 1.2 atmospheres of pressure.

After sowing, the jars were cultured for 10 days in an acclimatization room with 25°C and 16 hours light and 8 hours dark photoperiod. Seeds were checked every day and seeds with a rootlet length of 2 mm were considered germinated. Germination rate was calculated according to the equation of Matthews and Khajeh-Hosseini, 2007.

Germination Rate (%) = (Number of germinated seeds / Total number of seeds) ×100

Explant Preparation

Forage pea seeds were sterilized and left to germinate in MS medium for 7 days. The cotyledon and root nodes of the germinated seeds were cut with a scalpel and placed so that the cut point touched the solid regeneration medium. Explants were incubated in contact with MS nutrient medium containing phosphinothricin.

Determination of Root and Seedling Length

The roots and seedlings of the plants were cut with a scalpel after 10 days. The lengths of the roots and seedlings were measured with the help of caliper.

Measurement of Root and Seedling Wet Weight

The roots and seedlings of the plants were cut with a scalpel after 10 days. Each of the roots and seedlings were weighed on a precision balance and their wet weights were determined.

Evaluation of the Data

In the studies with explant, 50 explants for each replicate and 150 explants for a total of 3 replicates were cultured. The data obtained from the study were analyzed on the computer with SPSS 16 and MSTAT-C programs according to the factorial random plots experimental design and the means were compared with Duncan test.

3.RESULTS

According to the results obtained, the variation between the amount of phosphinothricin and plant growth parameters in vitro is given. As shown in Table 1, the doses had significant effects on germination rate, root length, shoot length, root wet weight and shoot wet weight ($p < 0.01$). Explant growth was not observed in phosphinothricin medium at different concentrations.

Figure 1.a. Cotyledon explants from phosphinothricin medium

Figure 1.b. Root node explants taken from phosphinothricin medium

Table 1. Statistical data examining the effect of different doses of phosphinothricin on various plant growth parameters

		Degree of freedom (df)	**Mean Square (MS)**	**F**
Germination	Doses	3	2041.667	98.000**
	Error	12	20.833	
	General	15		
Root length	Doses	3	21.075	76.986**
	Error	12	0.274	
	General	15		
Shoot length	Doses	3	24.038	613.507**
	Error	12	0.039	
	General	15		
Root wet weight	Doses	3	0.002	54.608**
	Error	12	0.000	
	General	15		
Shoot wet weight	Doses	3	0.006	462.251**
	Error	12	0.000	
	General	15		

According to the results of Duncan test in Tables 2, 3, 4, 5 and 6, dose 1.00 shows the highest values and dose 4.00 shows the lowest values. According to the results of all parameters, it is observed that the growth rates

decrease when the dose increases. As in Table 2, germination rate was calculated as 100.00 at dose 1.00, 82.50 at dose 2.00, 65.00 at dose 3.00 and 47.50 at dose 4.00.

Table 2. Germination rate according to Duncan test

Doses	**N**	Subset for alpha = 0.01			
		1	**2**	**3**	**4**
4.00	4	47.50 d			
3.00	4		65.00 c		
2.00	4			82.50 b	
1.00	4				100.00 a
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 4.000.

As shown in Table 3, root length was calculated as 6.80 cm at dose 1.00, 5.15 cm at dose 2.00, 4.63 cm at dose 3.00 and 1.33 cm at dose 4.00.

Table 3. Root length representation according to Duncan's test

Doses	**N**	Subset for alpha = 0.01		
		1	**2**	**3**
4.00	4	1.3250 c		
3.00	4		4.6250 b	
2.00	4		5.1500 b	
1.00	4			6.8000 a
Sig.		1.000	.181	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 4.000.

As shown in Table 4, shoot length was calculated as 5.65 cm at dose 1.00, 2.68 cm at dose 2.00, 0.61 cm at dose 3.00 and 0.35 cm at dose 4.00.

Table 4. Shoot length representation according to Duncan's test

Doses	**N**	Subset for alpha = 0.01		
		1	**2**	**3**
4.00	4	0.3500 c		
3.00	4	0.6075 c		
2.00	4		2.6750 b	
1.00	4			5.6500 a
Sig.		.091	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 4.000.

As shown in Table 5, root wet weight was calculated as 0.0673 g at dose 1.00, 0.0455 g at dose 2.00, 0.0363 g at dose 3.00 and 0.0150 g at dose 4.00.

Table 5. Root wet weight according to Duncan's test

Doses	**N**	Subset for alpha = 0.01		
		1	**2**	**3**
4.00	4	0.0150 c		
3.00	4		0.0363 b	
2.00	4		0.0455 b	
1.00	4			0.0673 a
Sig.		1.000	.046	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 4.000.

As shown in Table 6, the statistical significance level of shoot wet weight was 1%. P-values were calculated as 0.0170 (d), 0.0323 (c), 0.0600 (b) and 0.1063 (a).

Table 6. Shoot wet weight representation according to Duncan's test

Doses	**N**	Subset for alpha = 0.01			
		1	**2**	**3**	**4**
4.00	4	.0170 d			
3.00	4		.0323 c		
2.00	4			.0600 b	
1.00	4				.1063 a
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 4.000.

4. CONCLUSION, DISCUSSION AND RECOMMENDATIONS

The critical dose of phosphinothricin used as a selective marker in gene transfer was evaluated under in vitro conditions. According to the results obtained, germination rate, root and shoot length, root and wet weight of the plant decreased as the amount of phosphinothricin increased. When all parameters were evaluated, a statistically significant difference was found between the doses. Significant differences were found especially in morphological characteristics such as germination, root length, shoot length, root wet weight and shoot wet weight. In addition, no growth was observed when cotyledon and root knot explants were transferred to the medium. Prihatra et al., 2019 revealed that tomato explants did not grow in phosphinothricin medium. Naing et al., 2014 revealed that 0.3 mg/L PPT concentration strongly inhibited shoot regeneration of *Chrysanthemum*

plant explants. In gene transfer studies, the critical amount of phosphinothricin was determined as 2 mg/L since plant development will be achieved with the explant source.

The study shows that in vitro doses have a highly significant effect on plant growth parameters. It may be more useful to evaluate the study under field conditions to determine the appropriate dose range. It would be more appropriate to observe the effect of environmental conditions and the fertilizer, pesticide or other chemical used. The results obtained were carried out only in vitro.

REFERENCES

Cui, Y., Liu, Z., Li, Y., Zhou, F., Chen, H., & Lin, Y. (2016). Application of a novel phosphinothricin N-acetyltransferase (RePAT) gene in developing glufosinate-resistant rice. *Scientific Reports, 6:21259*, 1-11.

Das, A., Kumar, S., Nandeesha, P., Singh Yadav, I., Saini, J., Chaturvedi, S. K., & Datta, S. (2014). An efficient in vitro regeneration system of fieldpea (Pisum sativum L.) via shoot organogenesis. *Journal of Plant Biochemistry and Biotechnology, 23(2)*, 184-189.

Dmitrovi´c, S., Dragi´cevi´c, M., Savi´c, J., Milutinovi´c, M., Živkovi´c, S., Maksimovi´c, V., & ..... (2021). Antagonistic Interaction between Phosphinothricin and Nepeta rtanjensis Essential Oil Affected Ammonium Metabolism and Antioxidant Defense of Arabidopsis Grown In Vitro. *Plants, 10(142),*, 1-20.

Erdoğan, C. (2024). Evaluation of The Use of Plant Protection Products in Türkiye and in The World and Recommendations. *KSU J. Agric Nat 27 (2),*, 382-392.

Hussain, A., Ding, X., Alariqi, M., Manghwar, H., Hui, F., Li, Y., ... (2021). Herbicide Resistance: Another Hot Agronomic Trait for Plant Genome Editing. *Plants, 621*, 1-24.

Ivanov, S. (2019). Weeds and weed control in forage pea: A Review. *Agricultural Science and Technology, 11(2)*, 107 - 112.

Kantwa, S. R., Agrawal, R. K., Jha, A., Pathan, S. H., Patil, S. D., Choudhary, M., & Roy, A. K. (2019). Effect of different herbicides on weed control efficiency, fodder and seed yields of berseem (Trifolium alexandrinum L.) in central India. *Range Mgmt. & Agroforestry, 40 (2)*, 323-328.

Ludvikova, M., & Griga,, M. (2022). Pea transformation: History, current status and challenges. *Czech Journal of Genetics and Plant Breeding, 58(3),*, 127–161.

Matthews, S., & Khajeh-Hosseini, M. (2007). Length of The Lag Period Of Germination and Metabolic Repair Explain Vigour Differences In Seed Lots of Maize (Zea mays). *Seed Science and Technology, 35(1)*, 200-212.

Naing, A. H., Park, K. I., Lim , S. H., & Kim , C. K. (2014). Appropriate choice of antibiotics for plant regeneration and optimization of selective agents to be used in genetic transformation of chrysanthemum. *Plant Omics Journal 7(4),*, 237-243.

Prihatna, C., Chen, R., Barbetti, M. J., & Barker, S. J. (2019). Optimisation of regeneration parameters improves transformation efficiency of recalcitrant tomato. *Plant Cell, Tissue and Organ Culture, 137*, 473–483.

Sayar, M. S. (2021). Cultivation of Forage Pea and Important Agricultural Traits of GAP Pembesi Forage Pea Cultivar. *Dicle University Journal of the Institute of Natural and Applied Science, 10 (1)*, 85-94.