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Araştırma Makalesi/*Research Article (Original Paper)*

Alleviative Role of B-Estradiol Against 2,4-Dichlorophenoxyacetic Acid Genotoxicity on Common Bean Genome

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Abstract: 2,4-Dichlorophenoxyacetic acid (2,4-D) is one of the several herbicides that widely used to prevent development of *Taraxacum officinale* and broadleaf weeds on cereal crops culture. However, it is known that 2,4-D can cause genetic damage to plants at low concentrations as well as produce observable physiological effects. There is no report investigating the effect of mammalian hormones in crops against the applied 2,4-D. Therefore, the present study was aimed at investigating levels of DNA damage, changes in DNA methylation and DNA stability in common bean *(Phaseolus vulgaris)* exposed to 2,4-D and determine whether β-estradiol has any effect. RAPDs (Randomly Amplified Polymorphic DNA) and CRED-RAs (Coupled Restriction Enzyme Digestion-Random Amplification) techniques were used to define the DNA damage levels and changes in the pattern of DNA methylation. The obtained data demonstrated that 2,4-D led to an increase in RAPDs profile changes (DNA damage), and a reduction in genomic template stability (GTS). The effects caused by 2,4-D were decreased after application with different concentrations of β-estradiol. The results of this study clearly show that β-estradiol could be used function effectively to prevented from the genetic and epigenetic changes caused by 2,4-D herbicide in common bean.

Keywords: 2,4-D, DNA damage, DNA methylation, Genotoxicity, β-estradiol

Fasulye Genomunda 2,4-Diklorofenoksiasetik Asit Genotoksisitesine Karşı B-Östradiolün Hafifletici Rolü

Özet: 2,4-Diklorofenoksiasetik asit (2,4-D) tahıl ekin kültüründe *Taraxacum officinale* ve geniş yapraklı yabancı otların gelişmesini önlemek için yaygın olarak kullanılan herbisitlerden biridir. Ancak, 2,4-D'nin, düşük konsantrasyonlarda bitkilerde genetik hasarın yanı sıra gözlemlenebilir fizyolojik etkilere neden olduğu bilinmektedir. Uygulanan 2,4-D'ye karşı bitkilerde memeli hormonlarının etkisini araştıran herhangi bir rapor bulunmamaktadır. Bu nedenle, bu çalışma 2,4-D'ye maruz bırakılan fasulye (*Phaseolus vulgaris*)'deki DNA hasarı, DNA metilasyonu ve DNA stabilitesindeki değişimleri araştırmayı ve β-östradiolün herhangi bir etkisinin olup olmadığını belirlemeyi amaçlamıştır. DNA hasar düzeylerini ve DNA metilasyon modelindeki değişiklikleri tanımlamak için RAPD (Rastgele Çoğaltılan Polimorfik DNA) ve CRED-RA (Çift Restriksiyon Enzim Kesimi-Rastgele Çoğaltımı) teknikleri kullanılmıştır. Elde edilen veriler, 2,4-D'nin RAPD profil değişikliklerinde (DNA hasarında) artışa ve genomik kararlılık stabilitesinde (GTS) ise azalışa neden olduğunu göstermiştir. 2,4-D'nin neden olduğu etkiler, farklı β-östradiol konsantrasyonları uygulandıktan sonra azalmıştır. Bu çalışmanın sonuçları, fasulyelerde 2,4-D herbisitin neden olduğu genetik ve epigenetik değişikliklerin engellenmesi için β-östradiolün etkin bir şekilde kullanılabileceğini açıkça göstermektedir.

Anahtar kelimeler: 2,4-D, DNA hasarı, DNA metilasyonu, Genotoksisite, β-östradiol

Introduction

Phytoestrogens or plant-based estrogens occur from non-steroidal plant compounds. Especially again fungi, they are an important part of plant defence system (Leegood and Lea 1998). β-estradiol is similar in structural to phytoestrogens. β-estradiol has antiestrogenic and estrogenic effects and antiestrogenic effects show by blocking the receptor sites against estrogen (Yildiz 2005). Phytoestrogens show their impacts by interacting to estrogen

receptors (Turner et al. 2007) and modifiying the concentration of endogen-derived estrogens throught inactivating or binding enzymes. In addition to these, they have the ability to change bioavailability of sex hormones by blocking the synthesis of sex hormone-binding globulin (Johnston 2003). Because of similar properties estrogen and phytoestrogen allow sometimes act as estrogen antagonists against estrogens. Some studies proved that phytoestrogens have a protective effect opposite to variety disorders (Adlercreutz 2002; Johnston 2003).

Additionally, a few studies have demonstrated that β-estradiol, progesterone and androsterone that are exogenous mammalian sex hormones (MSHs) have positive impacts on plant growth and development inducing the activities of oxidative enzymes and synthesis reactions, reducing hydrogen peroxide (H_2O_2) content and lipid peroxidation (MDA) levels by blocking the activities of antioxidant enzymes, increasing contents of protein and nucleic acid, affecting the inorganic constituents of plants under non-stress conditions (Erdal and Dumlupinar 2011a; Dogra and Thukral 1996). Moreover, Erdal (2012) first recorded that MSH treatment eliminated decreasing superoxide dismutase (SOD), peroxidase (POX), nitrate reductase (NR) activities, ascorbate peroxidase (APX) and catalase (CAT), increased in the lipid peroxidation (MDA) level, superoxide (O_2^-) production and hydrogen peroxide (H_2O_2) content following salt treatment. The same researcher demonstrated that MSH treatment generated a preventive impact against effects caused by the amount of dry weight, chlorophyll, sugar, protein, glutathione (GSH) and proline stimulated by salt stress (Erdal 2012).

Despite these effects of β-estradiol, 2,4-D (2,4-Dichlorophenoxyacetic acid) has a negative influence on plant organisms. The processing principle of the molecular mechanism of 2,4-D mostly occurs in the form disruption of the hormonal equilibrium of the auxin-cytokinin system. In this wise, 2,4-D inhibits root and shoot growth in plants (Grabinsk et al. 2003). 2,4-D may disrupt many chemical reactions such as binding to the enzyme and change their activity, interacts with phospholipids, increase physical interactions in cell membranes (Bukowska 2006). The toxic concentrations of 2,4-D lead to apoptosis of cerebellar granule cells (De Moliner 2002). It is also devastated the vascular structure of thymus with fragmented DNA (Kaioumova 2001). There is some information about mutagenicity and genotoxicity of 2,4-D. The transgenic *Arabidopsis thaliana* was performed by point mutation and recombination tests the effect on depending 2,4-D. This study found a significant effect on the frequency of homologous recombination (Filkowski et al. 2003).

Many reports have been shown that the formation of an embryogenic cell is associated with nuclear DNA hypermethylation in the presence of 2,4-D (Lo Schiavo et al. 1989; Yoon et al. 1997; Leljak-Levanic et al. 2004). Pavokovi and Krsnik-Rasol (2012) reported that methylation observed influence cellular metabolism and glycation model of methylation observed in the sugar beet cells via a mechanism similar to animal cells (Pavokovi and Krsnik-Rasol 2012). Based on this information, it is known to be genotoxic effects of 2,4-D. However, these effects do not have any information about the amelioration with β-estradiol. The basis goal of the study was to detect whether β-estradiol has any protective effect against the negative effects of 2,4-D in *Phaseolus vulgaris*.

Materials and Methods

Common bean (*Phaseolus vulgaris* L. cv. Elkoca) seeds in sufficient size and amount were choosen and sterilized (0.5 % NaOCl -10 min and wash three times) for use in experiments. Seeds were exposed to β-estradiol at different concentrations (0, 10^{-6}, 10^{-7} and 10^{-8}M) for 96 h and were grown at 25±1°C in pots of a peat/soil mix (5 plants/pot) at with a 16 h photoperiod of 60 µmol photons/m^2s provided by white fluorescent lamp at a relative humidity of 70–75% until primary roots were grown at 0.5-1 cm length. Afterwards, the seedlings were sprayed at three-to-four-leaf stages with 2,4-D (5, 10, 20 and 40 mg/L) (number repetition: 3). Bulk leaves were randomly collected and were stored at -80°C. Genomic DNA was isolated from the seedlings using the method explained by Sigmaz et al. (2015) with minor modifications and stored at -20ºC. 32 RAPD primers that are ten-nucleotide length were tried with bulked control DNA (0 M β-estradiol + 0 mM 2,4-D). Only 14 out of 32 primers ((CAGAAGCGGA (OPW-4), TGTGGCAGCA (OPW-20), AAGGCTCACC (OPY-6, GGGCCAATGT (OPY-16), AATCGGGCTG (OPA-4), TGCCGAGCTG (OPA-2), TCTCAGCTGG (OPH-16), CAGGCCCTTC (OPA-1),) AGACGATGGG (OPY-11), CTGACCAGCC (OPH-19), GAATCGGCCA (OPH-18), AGGCCCGATG (OPW-6), GGGTCTCGGT (OPY-13), AGGCAGAGCA (OPY-8)) were used because of polymorphic amplicons in RAPD-PCR reactions.

PCR reaction contents and reaction conditions have been given in Table 1. PCR products were analyzed using 1% agarose gel electrophoresis and visualized with ethidium bromide staining.

Genomic DNA samples were digested with two restriction endonucleases (*HpaII* and *MspI)* and after checking digestion, amplification and visualization conditions for CRED-RA as defined for RAPD analysis. RAPD and CRED-RA patterns were analyzed by using the Total Lab (TL120) computer software (Yildirim et al. 2014).

Table 1. PCR reaction mix contents and conditions

PCR Reaction Mix Contents	
PCR buffer (10X)	1X
$MgCl_2$ (25 mM)	2.5 mM
dNTP (10 mM)	400 µM
gDNA	25 ng
Primer (100 pmol)	10 pmol
Taq DNA polymerase (5U/µl)	1 *U*
Total volume of each sample	20 µl
PCR Conditions	
1 cycle of 5 min at 95°C, 42 cycles of (1 min at 94°C, 1 min at 36°C and 2 min at 72°C), 1 cycles of 15 min at 72°C	

Results

In this study, both the RAPD and CRED-RA techniques were performed by applying the β-estradiol and 2,4-D concentrations on *Phaseolus vulgaris*. In the RAPD fingerprinting technique, the fourteen of the thirtytwo oligonucleotides giving amplification results was selected. Totally, the number of produced bands has been determined as 1265. Table 2 summarizes the RAPD results of selected primers. Each primer produces a band gap of 2-9 with an average of 4.75 bands per primer. According to the obtained results; amplified band was emerged between 216 and 2340 base-pairs in control seedlings. The differences shown among the control and treatment groups have given in Table 2. One of the primers (OPW-6) resulted no amplification products, others gave more complicated patterns of gains or losses. While the highest appearance of new bands was shown at OPA-1 primer, the highest disappearance of existing bands were shown at OPH-19 primer compared total treatment samples with the control samples. The polymorphic bands showed variability at each concentration of treated samples upon amplification with the primers used.

GTS value that was defined a quantitative measure of the genomic template stability changes in RAPD pattern was performed for fourteen primers at Table 2. In this table (Table 2), it is clear that the average GTS values decreased with the 2,4-D treatments. In addition, while the highest dose of β-estradiol increased the GTS rate, the other doses decrease its. As it is seen in the data, the highest genomic template stability rate value was obtained at 10^{-6} β-estradiol and the lowest rate value was obtained at 40 mg/2,4-D.

Eight primers gave polymorphic amplifications from among using the RAPD fingerprinting technique were chosen for application by the CRED-RA technique to state the effects of treated samples on methylation. The results of the CRED-RA method are presented as % of the mean polymorphism of DNA methylation for each concentration (Table 3). When the results obtained according to the CRED-RA technique are considered, the highest % polymorphism value was obtained at 40 mg/L 2,4-D (25.9%) and the lowest was obtained at 10^{-6} M β-estradiol (6.5%) doses. The concentrations of treated 2,4-D had an effect on methylation status.

Table 2. Molecular sizes (bp) of bands (+: appearance / -: disappearance) and the average GTS values in RAPD profiles.

Primers	C*	+/-	0 M β-estradiol+2,4-D (mg/L)				10^{-6} M β-estradiol+2,4-D (mg/L)					10^{-7} M β-estradiol+2,4-D (mg/L)					10^{-8} M β-estradiol+2,4-D (mg/L)				
			5	10	20	40	0	5	10	20	40	0	5	10	20	40	0	5	10	20	40
OPW-20	2	+	-	-	-	1000	-	-	1328	1342	1342	1200	1328	1242	1218	1242	1185	1271	-	-	-
		-	-	-	-	831	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPA-4	6	+	260	266	279	266	633; 234	247	247	216	485; 247	216	234	737; 200	216	709; 211	728	216	700; 353; 216	709; 154	689; 189
		-	-	-	-	-	-	-	-	-	579;	-	-	1537; 575	1537; 575	1537; 579	153; 575	1537; 579	1537	1537; 579	1537; 579
OPA-2	6	+	900	1000	911	900	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	865	1325; 1125	1325; 1125; 865	1325; 1125; 865	1325; 1125	1325; 1125	1325; 1125	1325; 1125; 648; 531	865	1125	1125	1125	1125	1125	1125	1125	1125	1125	1325
OPH-16	3	+	867	867	867	867	-	-	-	-	-	-	-	-	-	921	-	-	1788	1788	1800
		-	1655	1655	1655	1655	-	-	1344	1411	1411	-	1411	1377	1377	856	1355	1322	856	856	856
OPA-1	7	+	1820	1000	1780; 1060	1760	1000	-	1040	1840; 1000	1780; 1060,	1780;	1800;	1720; 978	1720; 978	1840; 1780 978	1060	1740; 1500	1780	1700	1040; 1700
		-	2340	2340; 768	-	768	768;	2340; 1840; 1040; 768	768	2340; 768	768	2340	2340	2340; 768	2340; 768	2340; 2140; 768	-	2340	2340; 2140	2340	2340
OPH-18	5	+	-	-	914	900	688	930	700	-	688	1200; 700	1616; 677	1866; 646	1183; 646	964; 646	677	430; 656	430; 618	875; 627	930; 618
		-	-	-	-	-	-	-	1866	1866	-	-	-	-	1866	-	-	-	-	-	-
OPY-6	5	+	-	1211	1211	1211	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	1622; 1366	1177	1177	1622		1622	1622	1622; 1366	1622; 1366;	1622; 1366	1622	1622	1622	1622	1622	1622	1622	1622	1622
OPY-11	4	+	973	973	634	1150; 1000	1050	1062	1025	-	1462	-	-	-	-	-	-	1050	-	1037	-
		-	654	634	675	948	-	-	1650	1650	1650	-	-	-	-	1650	1650	1650	1650	1650	1650
OPY-13	5	+	1675; 800	1675; 800	900	879; 478	-	-	853	-	809	-	-	559	827	657; 543	800	657; 521	870	853	827; 600; 356
		-	--	-	745	1325	-	-	1637	1637; 745	-	-	-	-	--	-	-	-	1637; 1325	1080	1637

(Table 2 continued)

OPH-19	3	+	562	562	537; 1080	1160 ; 949	-	-	-	745	1150	-	-	-	700	-	-	786	553	562	1350; 580
		-	610	670	-	1060	-	-	-	-	745	-	-	-	-	-	-	1712	1712	909	1860
OPY-8	7	+	-	-	-	-	-	-	-	-	986	959	1000	812	-	-	-	-	-	-	444
		-	1412	1525; 1412	1525; 1412	1525 ; 1412	1412	1525; 1412	-	1525	1525	1525	1525	1525	1712	1712; 1412	-	-	-	-	1712
OPY-16	4	+	951; 865	1350; 1000	1370; 841	1330 ; 1050	-	-	-	-	--	-	-	-	-	-	-	-	-	-	-
		-	1020 ; 769	1420; 769	1420; 769	1420 ; 769	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPW-6	6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OPW-4	4	+	1590	1660	1660	1660	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		-	423	423	423	423	423	423	423	423	423	423	423	423	423	423	423	423	423	423	423
GTS %			**63,8**	**60,6**	**59,7**	**48,0**	**87,1**	**83,8**	**75,9**	**69,8**	**68,3**	**82,4**	**81,5**	**74,4**	**72,8**	**66,8**	**80,4**	**71,6**	**70,6**	**71,4**	**63,4**

***: Control**

Table 3. CRED-RA band amounts and polymorphism %.

Primers	2,4 D (mg/L)	0 M β-estradiol		10^{-6} M β-estradiol		10^{-7} M β-estradiol		10^{-8} M β-estradiol	
		H	M	H	M	H	M	H	M
OPA-2	0	-	-		0	0	0	0	25
	5	16,6	14,2	33.3	0	12.5	12.5	14.2	12.5
	10	0	0	20	12.5	0	0	20	12.5
	20	20	14,2	0	0	37.5	0	16.6	25
	40	33,3	25	33.3	12.5	20	0	25	25
OPY-6	0	-	-	0	14.2	20	14.2	20	28.5
	5	60	0	20	28.5	11.1	0	20	0
	10	0	28,5	25	14.2	12.5	28.5	15.3	42.8
	20	33,3	28,5	40	0	0	14.2	20	42.8
	40	12,5	42,8	20	42.8	0	14.2	0	28.5
OPY-13	0	-	-	0	0	0	0	25	28.5
	5	0	20	11.1	0	20	14.2	0	14.2
	10	42,8	25	14.2	28.5	0	28.5	14.2	0
	20	40	0	16.6	42.8	0	28.5	11.1	0
	40	0	25	20	14.2	0	28.5	0	0
OPY-16	0	-	-	40	12.5	25	0	0	0
	5	0	40	0	0	25	0	11.1	25
	10	0	20	40	0	28.1	25	12.8	25
	20	33,3	50	11.1	25	20	0	0	12.5
	40	33,3	0	12.5	0	25	50	20	0
OPW-4	0	-	-	16.6	0	0	22.2	0	11.1
	5	16,6	20	0	22.2	0	11.1	16.6	0
	10	0	0	0	11.1	33.3	0	0	0
	20	25	20	0	0	0	33.3	0	11.1
	40	20	20	20	0	0	22.2	20	11.1
OPW-17	0	-	-	12.5	11.1	44.4	0	20	0
	5	33,3	20	25	22.2	0	33.3	17.8	14.2
	10	66,6	33.3	16.6	0	0	11.1	0	0
	20	0	0	0	0	25	11.1	17.8	28.5
	40	25	50	14.2	14.2	33.3	14.2	14.2	14.2
OPA-4	0	-	-	0	14.2	0	25	0	0
	5	25	25	0	0	0	20	0	20
	10	0	25	20	14.2	11.1	14.2	20	25
	20	0	33,3	12.5	28.5	0	14.2	20	0
	40	25	33,3	0	14.2	20	14.2	25	37.5
OPH-19	0	-	-	0	0	20	11.1	20	0
	5	33,3	0	14.2	22.2	12.5	22.2	0	22.2
	10	25	14,2	0	0	14.2	11.1	25	0
	20	0	0	0	11.1	0	0	16.6	33.3
	40	37,5	11,1	0	11.1	0	0	25	33.3
Average	**0**	**-**	**-**	**10.2**	**6.5**	**13.6**	**9**	**10.6**	**11.6**
	5	**23,1**	**17,4**	**12.9**	**11.8**	**11.8**	**14.1**	**9.9**	**13.5**
	10	**16,8**	**18.2**	**16.9**	**10**	**12.4**	**14.8**	**13.4**	**13.1**
	20	**18,9**	**18,2**	**10**	**13.4**	**10.3**	**12.6**	**12.7**	**20.7**
	40	**23,3**	**25,9**	**15**	**13.6**	**12.2**	**17.9**	**16.1**	**18.7**

Discussion

The 2,4-D is the herbicide used commonly on whole world, and it is a synthetic auxin plant hormone (Wauchope et al. 1992). The 2,4-D was observed influencing to various processes including the formation of free radicals, apoptosis, chromosomal aberrations, the inhibition of cell growth, protein and DNA synthesis, the formation of

hypermethylation on the plant (Almeira et al. 1995; Kaioumova 2001; De Moliner 2002; Grabinsk et al. 2003; Pavokovi and Krsnik-Rasol 2012). Moreover, genotoxicity and total soluble protein content were investigated by using RAPD, comet assay and SDS-PAGE, and herbicides (2,4-D and Dicamba) have genotoxic effects because of caused homologous recombination and the A → G point mutation increases at low concentration. Therefore, 2,4-D was showed to cause DNA damages. (Filkowski et al. 2003; Cenkci et al. 2010). Oakes and Pollack (2000) reported that 2,4-D may be connected with aminoacids (especially among plants) alanine, glutaminic and asparaginic acid, and also phenylalanine, tryptophane and isoleucyne (Oakes and Pollack 2000). Our study observed that 2,4-D caused DNA damages and decrease to GTS% rates.

There are many studies on MSHs. The studies demonstrated that exogenous application of mammalian sex hormones (such as β-estradiol, progesterone and androsterone) substantially improved plant growth and development, augmented protein and nucleic acid contents, stimulated oxidative enzyme activities, reduced H_2O_2 content, and the lipid peroxidation (MDA) level under non-stress conditions (Karl and Lauchli 2010; Erdal and Dumlupinar 2010). Also, MSHs also affected the inorganic constituents of plants (Erdal and Dumlupinar 2011a). The same researches reported that MSH treatment significantly increased the Na content in chickpea seeds and barley leaves. Other researches shown that although MSH treatment increased the Na, K and Ca contents in chickpea seedlings, they decreased the Cl content (Afzal et al. 2006; Erdal and Dumlupinar 2011b). Erdal (2012) reported that MSH application together with salt stress increased the dry weight, sugar, proline, protein, chlorophyll, glutathione (GSH) contents, superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), ascorbate peroxidase (APX), nitrate reductase (NR) activities, and reduced the lipid peroxidation (MDA) level, superoxide (O_2^-) production and hydrogen peroxide (H_2O_2) content compared with salinity alone. In the light of these findings, we applied β-estradiol to bean seeds under the influence of 2,4-D and observed to reduce the DNA damages by using the RAPD technique.

DNA methylation also plays an important role in plant development. In recent years, it has come to the conclusion that a great deal of genetic demethylation is a pleiotropic effect of plant morphology (Finnegan et al. 2000). Genome imprinting as known the differential expression of genes inherited from maternal and paternal genomes is interfered by differential methylation of the two genomes in endosperm (Vielle 1999). DNA methylation plays an integral role in controlling the activity of transposable elements and in introducing DNA fragments such as transgenic silencing (Kloti 2002). In a study performed, the changes in chromatin structure by DNA methylation at presence of 2,4-D leads to genomic reprogramming in somatic cells (Karani and Saidi 2010). The highest rate of DNA methylation in 2,4-D containing medium was observed and the DNA methylation in auxin containing medium was decreased in Cucurbita plant (Leljak-Levanic et al. 2004). In our study demonstrated that application of 2,4-D with β-estradiol was reduced DNA methylation change. these data suggest the protective role of β-estradiol under stress conditions. Thus, we thought that β-estradiol reduced the negative effects of 2,4-D. The previous studies showed that an overproduction of reactive oxygen species such as superoxide radicals (O_2^-) and hydrogenperoxide (H_2O_2) could take place in plants treated with 2,4-D (De Moliner 2002; Romero-Puertas et al. 2004). Reactive Oxygen Species (ROS) cause epigenetic changes without causing alteration in DNA sequence as well as causing changes in DNA sequence (Creppy et al. 2002; Marnet et al. 2003; Valinluck et al. 2004). On the other hand, several studies have shown that exogenous mammalian sex hormones such as progesterone, β-estradiol and andosterone affect hydrogen peroxide (H_2O_2) content and lipid peroxidation monodihydroascorbate (MDA) levels by stimulating the activity of antioxidant enzymes. So these hormones have positive effects on plant growth and development under stress (Dogra and Thukral 1996; Erdal and Dumlupinar 2011b). The obtained results were thought that the protective role of β-estradiol may be from the reduced ROS levels and the effect of ROS causing epigenetic changes. According to our knowledge, the present study represents the first data demonstrating the impacts on DNA methylation of β-estradiol under non-stress and exposure of 2,4-D in plants. β-estradiol application to may be useful for large-scale agricultural benefit, and we intend to actualize such an investigation in the near future.

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