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Research Article / Araştırma Makalesi

Determination of Pesticide-Induced Genotoxicity on Soybean (*Glycine max* L.)

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Abstract: Pesticides are used in agriculture and cause side effects in plants and can be transported to products which we consume. Genotoxic chemical substances distributed to environment and higher plants such as *Glycine max* have been used as an indicator plants that show the genotoxic effects of environmental chemical pollutants. In this respect we investigated the potential genotoxic effect of three different pesticides (Pomarsol Forte WP 80 as a fungucide, Arrivo 25 EC as an insecticide, and The End EC as an herbicide) on *G. max* (*Glycine max* L.) for the first time. In order to determine the genotoxic effects of these pesticides on *G max*. Median EC (effective concentration) determination analysis, RAPD-PCR (randomly amplified polymorphic DNA-polymerase chain reaction) assay and protein analysis were used. Our results indicated that The End as a herbicide had more inhibitory effects on *G. max* root growth compare to the other pesticides. 20 RAPD primers were used, eighteen primers gave stable results while 11 of them were polymorphic and 7 of them showed the same band profile. Percentage of polymorphism was found as 20%. Total protein content was significantly decreased by insecticide treatment but increased in herbicide treatment ($p<0.05$). In conclusion these results suggest that these pesticides have genotoxic effects on *G. max* and the use of these chemicals must be reduced to avoid exposure to humans and the environment.

Keywords: cypermethrin, quizalofop-p-ethyl, RAPD-PCR, SDS-PAGE, thiram.

Pestisit-Kaynaklı Genotoksisitenin Soya Fasulyesinde (*Glycine max* L.) Belirlenmesi

Öz: Pestisitler, tarımsal alanlarda yaygın olarak kullanılırken bitkilerde yan etkilere neden olmaktadır ve tükettiğimiz ürünlere de taşınabilmektedir. Genotoksik kimyasal maddeler çevreye yayılmaktadır ve *Glycine max* gibi yüksek yapılı bitkiler, kimyasal çevre kirleticilerinin genotoksik etkilerini gösteren indikatör bitkiler olarak kullanılmaktadır. Bu bağlamda, mevcut çalışmada ilk kez üç farklı pestisitin (fungusit olarak Pomarsol Forte WP 80, insektisit olarak Arrivo 25 EC ve herbisit olarak The End EC) potansiyel genotoksik etkisi soya fasulyesi (*Glycine max* L.) üzerinde araştırılmıştır. *G. max* üzerindeki genotoksik etkiyi belirlemek için ortalama etkili konsantrasyon (median EC), RAPD-PCR (rastgele amplifiye polimorfik DNA-polimeraz zincir reaksiyonu) ve protein analizleri kullanılmıştır. Elde edilen sonuçlar bir herbisit olarak The END pestisitinin diğerlerine göre *G. max* kök büyümesi üzerinde daha fazla inhibe edici etkisi olduğu saptanmıştır. 20 RAPD primeri kullanılmış; bunlardan 18 primer stabil sonuç verirken, 11 tanesi polimorfik ve 7 tanesi benzer bant profili göstermiştir. Polimorfizm yüzdesi, % 20 olarak bulunmuştur. Toplam protein içeriği insektisit muamelesi ile kontrol grubuna göre belirgin şekilde azalırken, herbisit muamelesi sonrası anlamlı olarak artmıştır ($p <0.05$). Sonuç olarak elde edilen veriler bu pestisitlerin *G. max* üzerinde belirgin genotoksik etkilerinin olduğunu ve bu kimyasallara karşı insan ve çevresel maruziyetin atmasından dolayı kullanımdan kaçınılması gerektiğini göstermektedir.

Anahtar kelimeler: cypermethrin, quizalofop-p-ethyl, RAPD-PCR, SDS-PAGE, thiram.

1. Introduction

Numerous pesticides are widely used in agriculture and their usage has become required; however, incorrect uses of these chemical substance causes side effects in plants (Aksoy, Dana, Sanal, & Aktaç, 2007). Pesticides are used in different combinations, at different growth times and storage stages to protect agricultural products from harmful organisms (such as fungi) and to obtain high quality products. Pesticides accumulated after processing in food can be transported to products such as baby food (Wang, Chang, Hwang, Turnbull, & Howard, 2000).

The dithiocarbamate derivative thiram (the active ingredient of Pomarsol Forte 80 WP) is widely used in agriculture to protect vegetables and prevent mold contamination Cypermethrin (the active ingredient of Arrivo 25 EC) is a synthetic pyrethroid used to protect the consumption of commercial agricultural land and household products from insects. Quizalofop-p-ethyl (active ingredient of The End) is a phenoxy herbicide used for controlling weeds and in many agricultural lands like lentils (Villani, 1998).

The developments in industry and economy have caused the production of genotoxic chemical substances distributed to the environment which cause harmful effects and even death for human beings Poli et al., 1999). Higher plants have been used as an indicator plants that show the genotoxic effects of environmental chemical pollutants (Angelis, McGuffie, Menke, & Schubert, 2000; Yıldız & Arıkan, 2008; Yıldız, Ciğerci, Konuk, Fidan, & Terzi, 2009).

Evaluating the genotoxic effect through the DNA is more useful because it produces the results in a short time and give precise results. The use of DNA-based techniques to detect changes in DNA sequences is is becoming widespread. Randomly amplified polymorphic DNA (RAPD) is a method that can be used in genotoxicity studies and changes in the RAPD band profile clearly indicate changes betweeen treated and untreated groups

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in DNA levels against genetic agents (Martins, Lopes, Brehm, & Ribeiro., 2005; Enan, 2006; Liu et al., 2007). In addition, the amount of DNA damage and mutations in bacteria, plants, and animals can be determined through genomic DNA by RAPD as a molecular marker technique (Atienzar, Conradi, Evenden, Jha, & Depledge, 1999). Thus, the genotoxic effect of toxic chemicals has been regarded to alter genomic template stability through the changes in RAPD band profile (Wang, Lu, & Shen, 2007; Cenkci et al., 2010).

Our study is aimed to analyze the effect of pesticide pollution on the genetic material of *G. max* by use of the RAPD-PCR and to analyze the alterations in RAPD band profiles with respect to total protein levels and SDS-PAGE band profiles of whole seed proteins. *G. max* was used in this study as an experimental material as it is an important agricultural plant worldwide. We thought that obtained results may suggest a tolerable level of toxicity on *G. max* against to these pesticides which are used widely in agriculture.

2. Materials and Methods

2.1. Chemicals

Pomarsol Forte WP 80 (thiram), [bis(dimethylthiocarbamoyl) disulfide] is a type of sulfur fungicide which is a 80% water-wettable powder produced by Bayer Company, Arrivo 25 EC (cypermethrin), [(RS)-α-cyano-3-phenoxybenzyl (1RS)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate] is a synthetic pyrethroid which is a 25% emulsifiable solution produced by Hektaş Company and The End EC (Quizalofop-p-ethyl), [Ethyl (2R)-2-[4-(6-chloroquinoxalin-2-yloxy)phenoxy]propionate] is a phenoxy herbicide which is a 5% emulsifiable solution by Agrogeneral Company were used as pesticides in this study.

2.2. Determination of EC_{50} and Treatment of *G. max* Seeds with Pesticides

G. max seeds (50 smiliar pieces) were germinated in distilled water for 24 h and treated with ten different concentrations of Pomarsol Forte WP 80 (0.00125, 0.0025, 0.005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 M), Arrivo 25 EC (0.075, 0.15, 0.3, 0.6, 1.2, 2.4, 4.8, 9.6, 19.2 ve 38.4 M) and The End (0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 ve 6.4 M) for 72 h to determine EC_{50} (Effective concentration 50). The control group was only treated with distiled water. The root lengths were measured and T/C% was calculated after treatment for 72 h. After determination of EC_{50} seeds of *G. max* were treated with EC_{50} and $2xEC_{50}$ concentrations for each pesticide. When the roots were 0.5 cm or higher, it was accepted as germinated.

2.3. Randomly Amplified Polymorphic DNA- Polymerase Chain Reaction (RAPD- PCR)

G. max roots were grinded in liquid nitrogen and DNA was isolated with DNeasy Plant Mini Kit (Qiagen). DNA concentration was measured at 260/280 nm using spectrophotometrically. Twenty primers (Table 1) were used in RAPD-PCR assay and optimized to Williams, Kubelik, Livak, Rafalski, and Tingey (1990). In order to determine the molecular weights of the RAPD-PCR amplicons 100-bp, DNA ladder was used. An UV imaging device was used to examine the RAPD-PCR band profile and the Vision WorksLS (Version 6.8) software was used to calculate the each amplicon size. Amplification products were scored as presence (1) or absence (0) and only strong bands were used for analysis. Genetic similarity coefficients between the treatment and untreated control groups were calculated according to Nei (1978)'s unbiased measure via POPGENE (version 1.31) software. Genomic template stability (GST) was estimated using the formula of "GST% = (1-a / n) X 100" (a: RAPD polymorphic bands, n: total bands of control).

Table 1. List of primers name (ID) and their nucleotide sequences used in the RAPD analysis

Number of primers	Name of primers	Sequences of primers (5′ → 3′)	Tm (°C)	Rate of GC %
1	AD1	GTTGCGATCC	32	60
2	AD2	GTGCCTAACC	32	60
3	AD3	ACGCGCATGT	32	60
4	AD4	GACGCCACAC	34	70
5	AD5	CCAGCTTAGG	32	60
6	AD6	CCCGCTACAC	34	70
7	AD7	GAGCGTCGAA	32	60
8	AD8	TGCGAGAGTC	32	60
9	AD9	CAGCCCAGAG	34	70
10	AD10	TCGCCGCAAA	32	60
11	AD11	GGCACGTAAG	32	60
12	AD12	CCCAGTCACT	32	60
13	AD13	TCGGCGGTTC	34	70
14	AD14	CCATTCCCCA	32	60
15	AD15	ACAGGTGCGT	32	60
16	AD16	GGACGACAAG	32	60
17	AD17	CAGAGGTCCC	34	70
18	AD18	TCCGATGCTG	32	60
19	AD19	GTCGTTCCTG	32	60
20	AD20	AAAGGGGTCC	32	60

2.4. Protein Analysis

Seed storage total soluble protein isolation was done as described by Saraswati, Matoh, Phupaibul, Lumpkin, & Kobayashi (1993) after 72 h of incubation. Following protein extraction, the Bradford Assay method was used to analyze the concentrations of the total soluble proteins present in the seed (Bradford, 1976). The SDS-PAGE of total seed protein was done as described by Laemmli (1970). After SDS-PAGE performed, the gel was visualized using a photo imaging system and the dendrogram was generated by Visionworks (Version 6.8) software based on the presence or absence of polypeptide bands on the SDS-PAGE gel.

2.5. Statistical Analysis

The data obtained was statistically analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used and p-values less than 0.05 are considered ''statistically significant.''

3. Results and Discussion

EC_{50} values were determined ~0.08 M for Pomarsol Forte WP 80 fungicide, ~9.6 M for Arrivo 25 EC insecticide, and ~0.4 M for The End herbicide. Thus, experiments were done using 0.08 M (EC_{50}) and 0.16 M ($2xEC_{50}$) Pomarsol Forte WP 80 concentrations, 9.6 M (EC_{50}) and 19.2 M ($2xEC_{50}$) Arrivo 25 EC concentrations and 0.4 M (EC_{50}) and 0.8 M ($2xEC_{50}$) The End concentrations. It was indicated that The End herbicide had more inhibitory effects on *G max*. seedlings compare to the Pomarsol Forte WP 80 fungicide and Arrivo 25 EC insecticide.

The End herbicide, Pomarsol Forte WP 80 fungicide, and Arrivo 25 EC insecticide are widely used in *G. max* agricultural applications. The genotoxic effects of these pesticides on *G. max* were investigated for the first time and their possible genotoxic effects were determined. Also studies involving possible genotoxic effects of pesticides on plants are limited to several plant species and need to be expanded.

In order to determine the genotoxic effects of different three pesticides, the RAPD analysis was used between treated and untreated groups with pesticides. Twenty primers (60–70% GC content) were used for evaluating the changes on genomic DNA. Eighteen primers gave stable results while 11 of them were polymorphic and 7 of them showed the same band profile. Additionally, 11 polymorphic RAPD primers showed differences (disappearance and/or appearance) in band profiles between pesticides treated and untreated groups (Fig. 1). Totally 55 bands of untreated group and 308 bands of treated groups were obtained ranged from 146 to 1077 bp. RAPD profile changes of treated and untreated groups were shown in Table 2 compared with their control groups. Value of polymorphisms P (%) was found 20%. It was suggested that the changes in RAPD profile as in Fig. 1, polymorphism was due to the loss and/or gain of the bands in pesticide treated groups compared to the control RAPD band profiles. Obtained results indicated that DNA damage may be serious in the *G. max* root cells after the treatment of these pesticides. When we consider the reasons for the loss or gain of the band causing polymorphism, it can be seen that these conditions may be due to breaks in DNA, modified bases, bulky adducts, DNA-protein cross-links or point mutations (Atienzar Venier, Jha, & Depledge, 2002; Wolf, Blust, & Backeljau, 2004). Appearance and disappearance of bands were found in our RAPD profiles in pesticide treated groups and it can be attributed to mutations and DNA damage on *G. max* (Atienzar & Jha, 2006).

Table 2. The number of bands in control and molecular sizes (base pair, bp) of disappearance (-) and/or appearance (+) of DNA bands for all primers of pesticide-treated germinated root tips of soybean using UVI soft image analyzer software.

			Pomarsol Fotre WP 80		Arrivo 25 EC		The End EC	
Primers	**Control**		**PX**	**P2X**	**AX**	**A2X**	**TX**	**T2X**
AD-1	5	-	636	636	0	0	0	0
		+	848; 471	848	1077; 848; 636; 351	1077; 848; 351	381; 906; 1077	381; 906; 1077
AD-3	5	-	0	0	0	0	0	0
		+	0	0	825	0	0	0
AD-4	6	-	854	854	626	626	854	345; 420; 854
		+	0	0	0	854	0	0
AD-5	3	-	0	0	325	325	0	0
		+	0	0	0	0	0	0
AD-7	5	-	0	0	0	357; 488	0	0
		+	0	0	0	0	396	396
AD-8	6	-	0	0	0	422	276	276
		+	0	0	0	0	715; 833	715; 833
AD-10	5	-	0	0	325	252; 325	0	0
		+	0	0	0	628	1264	0
AD-13	3	-	0	1086	0	0	146	146
		+	0	0	0	447; 538	0	0
AD-15	7	-	0	0	418; 454	418; 454	0	0
		+	0	0	0	0	0	0
AD-17	5	-	0	0	0	0	0	0
		+	385	385	0	0	0	0
AD-20	5	-	0	0	252	252	248	248
		+	0	769	0	0	0	0
Total	55		2 (-);3(+)	3 (-);3 (+)	6 (-);5(+)	7 (-);10(+)	4(-);7(+)	6 (-);6(+)

*PX: 0.08 M, P2X: 0.16 M, AX: 9.6 M, A2X: 19.2 M, TX: 0.4 M and T2X: 0.8 M

Additionally, GTS values were calculated for each polymorphic 11 primers and shown in Table 3. The average highest decrease in GTS value (76.9%) was calculated in 19.2 M Arrivo 25 EC insecticide treatment and GTS values were decreased in all the highest pesticide treatment compared to the untreated control groups. DNA damage, success in repair, and replication of DNA are related in GTS value. Our results showed that GTS values were decreased in all pesticide treatments compared to the untreated control groups. However we cannot say that it is related to the high DNA damage of pesticides because efficacy of DNA repair and DNA replication are

suppressed via the high level of DNA damage (Atienzar & Jha, 2006).

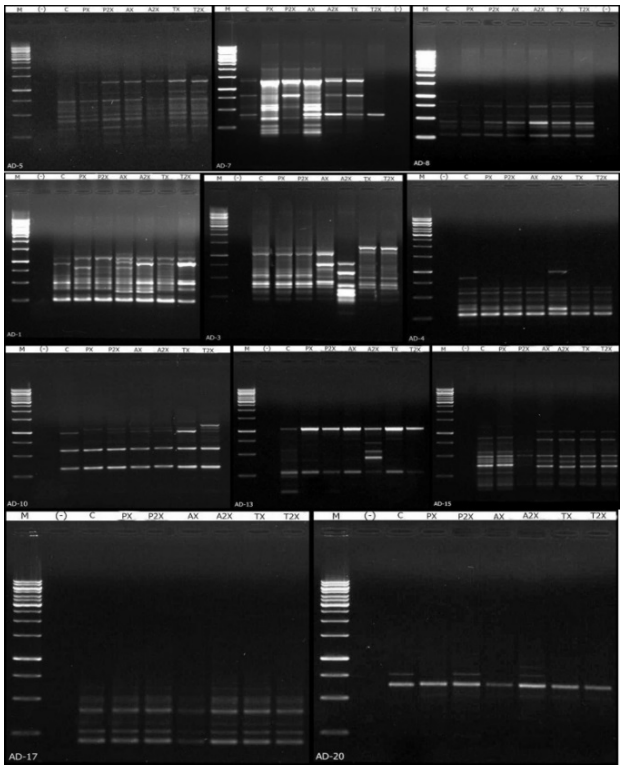

Figure 1. Polymorphic RAPD profiles of pesticide-treated germinated root tips of soybean generated using with primer AD-1, AD-3, AD-4, AD-5, AD-7, AD-8, AD-10, AD-13, AD-15, AD-17 and AD-20. [C: Control, PX: 0.08 M Pomarsol Forte 80 WP, P2X: 0.16 M Pomarsol Forte 80 WP, AX: 9.6 M Arrivo 25 EC, A2X: 19.2 M Arrivo 25 EC, TX: 0.4 M The End EC, T2X: 0.8 M The End EC, M: GeneRuler 100 bp plus DNA Ladder (100–3000 bp)].

The band profiles of *G. max* seeds exposed to different pesticides after SDS-PAGE were shown on Fig. 2. After the treatment of different pesticides, there is a statistically significant reduction in insecticide treatment but a significant increase in herbicide treatment in protein amount of *G. max* ($p<0.05$) (Fig. 3). The dendogram obtained by the evaluation of all protein bands after SDS-PAGE analysis and the genetic distance values were shown on Fig. 4. SDS-PAGE analysis showed that there were changes in protein profiles between the different pesticide treatments groups compared to the untreated control group.

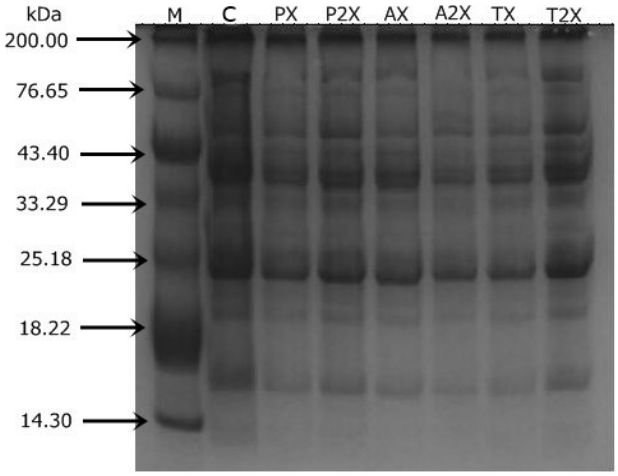

Figure 2. The band profiles of soybean seeds exposed to different pesticides after SDS-PAGE (M: Marker C: Control, PX: 0.08 M Pomarsol Forte 80 WP, P2X: 0.16 M Pomarsol Forte 80 WP, AX: 9.6 M Arrivo 25 EC, A2X: 19.2 M Arrivo 25 EC, TX: 0.4 M The End EC and T2X: 0.8 M The End EC)

It is known that total protein content of cells is an important indicator of various changes in metabolism and changes in response to various environmental pollutants (Singh & Tewari, 2003). It has been showed that various pesticides reduce total protein content in various organisms like *Brassica juncea* L. or *Aporrectodea caliginosa* (Singh & Tewari, 2003; Moshley, Ismail, & Ahmed, 2003). In this study, changes in total protein content of *G. max* roots treated with different pesticides showed an inverse relationship with the pesticide type and we thought that this change is a respond to these pesticides that were used.

Table 3. Genomic template stability (GTS, %) values.

		Pomarsol Forte 80 P		Arrivo 25 EC		The End EC	
Primers	**Control**	**PX**	**P2X**	**AX**	**A2X**	**TX**	**T2X**
AD-1	100	40.0	60.0	100	100	100	100
AD-3	100	100	100	80.0	100	100	100
AD-4	100	83.3	83.3	83.3	66.6	83.0	50.0
AD-5	100	100	100	66.6	66.6	100	100
AD-7	100	100	100	100	40.0	60.0	60.0
AD-8	100	100	100	100	83.3	50.0	50.0
AD-10	100	100	100	80.0	40.0	80.0	100
AD-13	100	100	66.6	100	100	83.3	83.3
AD-15	100	100	100	71.6	71.6	100	100
AD-17	100	80.0	80.0	100	100	100	100
AD-20	100	100	80.0	80.0	80.0	80.0	80.0
Average	**100**	**91.1**	**88.0**	**87.2**	**76.9**	**85.0**	**83.9**

*PX: 0.08 M, P2X: 0.16 M, AX: 9.6 M, A2X: 19.2 M, TX: 0.4 M and T2X: 0.8

In conclusion, plants are directly exposed to pesticides as well as soil, water, and air. Moreover, pesticides are very reactive molecules and they can modify the cellular structures, especially DNA. The present finding supports the claim that these pesticides made a genetic modification on *G. max* seeds and roots. Pesticides have toxic effects on human and the environment, because they accumulate on plants and show toxic effects, exactly resulting with DNA damage. Pesticides have a wide range of usage in different *G. max* and other plants on agricultural areas and these are discharged into the environment, plants, humans, and so into ground waters. Pesticides are very important due to the mutagenic effects on plants and humans. Therefore, the usage of pesticides should be considered carefully and reduced in order to decrease their exposure to humans and environment.

Further molecular studies are needed to evaluate the genotoxic effects of these chemicals.

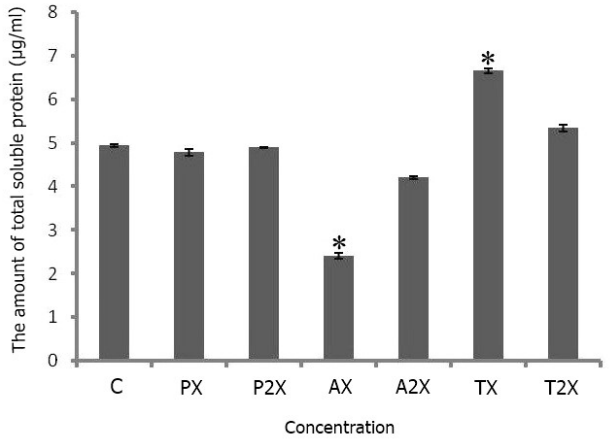

Figure 3. The effects of different pesticide applications on total protein amount in soybean seeds. The values are significantly different at $p < 0.05$ compared with the control group. (PX: 0.08 M Pomarsol Forte 80 WP, P2X: 0.16 M Pomarsol Forte 80 WP, AX: 9.6 M Arrivo 25 EC, A2X: 19.2 M Arrivo 25 EC, TX: 0.4 M The End EC, T2X: 0.8 M The End EC).

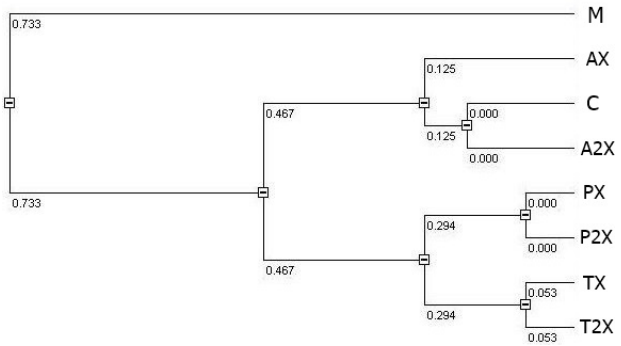

Figure 4. The dendogram obtained by the evaluation of all protein bands after SDS-PAGE (M: Marker, C: Control, PX: 0.08 M Pomarsol Forte 80 WP, P2X: 0.16 M Pomarsol Forte 80 WP, AX: 9.6 M Arrivo 25 EC, A2X: 19.2 M Arrivo 25 EC, TX: 0.4 M The End EC, T2X: 0.8 M The End EC)

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