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MOLECULAR BASIS OF RESISTANCE TO CLODINAFOP-PROPARGYL, AN ACETYL-COA CARBOXYLASE INHIBITING HERBICIDE, IN GREEN ALGAE *Scenedesmus quadricauda*

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ABSTRACT

Mutations in chloroplastic acetyl-CoA carboxylase (ACCase) gene enables molecular tools such as allele-specific PCR assay to monitor resistance alleles in green algae (Chlorophyta) *Scenedesmus quadricauda*. An isoleucine-leucine substitution in the gene encoding chloroplast (ACCase) conferred resistance to clodinafop-propargyl herbicide. Green algae cultures were treated with different concentrations of this herbicide (0, 1/16, 1/8, 1/4, 1/2, 1 and double of field concentration). The free amino acid content and cell number were determined after 0, 24, 48, 72 and 96 hrs.

Concerning cell number, from the first to third generation, the number of cells decreased especially in the highest two concentrations. From the fourth to sixth generation the number of cells increased in all tested concentrations except the two highest concentrations. With regard to amino acid content, results indicated that from the first to sixth generation an increase occurred in amino acid content 24 hrs after exposure and decreased 48-96 hrs after exposure. In the fourth and fifth generation amino acid content increased, while in the sixth generation decreased. That might be explained by the recovery of algae activity at the sublethal concentrations, emergence of algae resistant population as well as increase in algae cell number. The results of allele-specific PCR revealed the presenence of (C) allele in algal cultures which explain the resistance to the herbicide used.

Keywords:herbicides resistance, clodinafop-propargyl, acetyl-CoA carboxylase, mutation, *Scenedesmus quadricauda*

INTRODUCTION

Acetyl-CoA carboxylase catalyzes the first committed step in the biosynthesis of fatty acids. The enzyme is found in all animals, plants and bacteria, and catalyzes the biotin-dependent carboxylation of acetyl-CoA to form malonyl-CoA in two steps (Blanchard and Waldrop, 1998). The herbicides inhibiting acetyl-CoA carboxylase (ACCase) are widely used in agriculture, since they provide selective and effective weed control. However, the frequent use of herbicides in large quantities, usually leads to soil contamination and subsequent pollution of surface water and groundwater (Breton, *et al.*, 2006).

Moreover, the use of herbicides is threatened by the emergence of resistant biotypes (Amanda *et al.*, 2002) that considerably decreases the efficacy of herbicide treatments. With the increasing development of weed resistance to many popular selective herbicides, the need has arisen to diagnose herbicide resistant weeds as a first step in resistance management and monitoring their nature, distribution, and effective screening tests (Becki *et al.*, 2000).

Herbicide resistance may occur as a result of one or more mechanisms including reduction in herbicide uptake or translocation, increased herbicide metabolism, sequestration of the herbicide or modified target site (Maneechote *et al.*, 1994). Molecular basis of resistance is often a mutation at the site of action of herbicide in target enzyme or protein (Devine and Shimabukuro, 1994). In most of weed biotypes, resistance to ACCase inhibitors is conferred by reduced sensitivity to these herbicides. In resistant biotypes of *Lolium multiflorum*, resistance is conferred by tolerant form of ACCase. ACCase activity measured in extracts from etiolated shoots of a resistant biotype was found to be 28 fold more tolerant to diclofop than that from susceptible biotypes (Gronwald *et al.*, 1992). A single isoleucine-leucine substitution at herbicide-binding site in ACCase discriminated sensitive and resistant lines (Zagnitko *et al.*, 2001 and Délye *et al.*, 2002).

Many ACCase herbicide resistant biotypes populations of *L. rigidum* resistant to ACCase inhibiting herbicides is due to a resistant ACCase enzyme. Allele-specific PCR results further confirmed the mutations linked with resistance in these populations. The isoleucine-leucine substitution at position 1781 and Gln-Glu substitution at position 1756 has been identified in resistant grass specices (Zhang and Powles, 2006).

Screening for mutations related to the presence of herbicides in the aquatic environment can reveal biological effects, particularly adaptation at the genetic level, map-based approaches, genomic sequencing and functional genomics that may play vital roles in understanding the molecular basis of resistance to herbicides (Abou-Waly *et al.*, 1991 and Basu *et al.*, 2004). In this work, simple method based upon allele specific polymerase chain reaction (PCR) to detect an isoleucine-leucine substitution in the gene encoding chloroplastic acetyl-CoA carboxylase (ACCase) Délye, *et al.* (2002) were used to conferred resistance to the herbicides in green algae (Chlorophyta) *Scenedesmus quadricauda*. In addition, free amino acid content and cell number were determined as a resistance mechanisms.

MATERIALS AND METHODS

Culture conditions

Green algae (Chlorophyta) *Scenedesmus quadricauda* (strain Berb 614) was kindly supplied by Faculty of Science, Assiut University, Egypt. The medium for the algal growth was prepared according to Modified Bristol's Medium (MBM), (Wong, 2000). Algal were propagated photoautotrophically in a 500 ml Erlenmeyer round flasks supplemented with compressed air (to prevent cells from clumping) and continuous illumination by cool-white fluorescence lamps giving approximately 3000 Lux. The trial was conducted at room temperature (23±4°C).

Herbicide treatments

Clodinafop, aryloxyphenoxypropionate (APP) herbicide, a selective postemergence and foliar absorbed, was used in this study. The herbicide in wettable powder form was diluted with sterile distilled water and added into the sterile MBM in various concentrations (calculated as ppm active

ingredient). Cells were exposed to the herbicide concentarations: 336 (2X), 168 (X), 84 (1/2X), 42 (1/4X), 21 (1/8X) and 10.5 (1/16X) ppm, where (X) is the field concentration. The phytotoxicity assay started with a homogenous population of *Scenedesmus quadricauda* cells at the beginning of the cell cycle. Data were recorded after 0, 24, 48, 72 and 96 hrs as exposure periods.

Determination of cell number

Five ml of the culture samples were diluted with 1 ml of 10% formaldehyde solution. One drop of the algal suspension was pipetted on a slide (Haemocytometer, 0.1 mm deep, A.O. Spencer "Bright Line"), then coverd and left for 2 min for algal setting. The mean counts of three replicates were calculated ($x10^4$).

Determination of free amino acids

Free amino acids were determined according to (Lee and Takahashi, 1966). In a test tube, one ml of aliquot of sample extract was added. The tubes were then capped and inserted in a boiling water bath for 20 min. The tubes were then removed, and 5 ml of diluent solvent were added, mixed well immediately. The developed blue violet color was measured by a spectrophotometer (JENway 6405 UV/ vis Germany) at 570 nm.

PCR analysis conditions

PCR analysis was done using DNA of 5 algal samples. The PCR mixture and amplification conditions were prepared according to Williams *et al.*, (1990); Wink, and Wehrle (1994) with minor modifications.

A method based upon allele-specific PCR to detect an isoleucine-leucine substitution in the gene encoding chloroplastie-acetyl-CoA carboxylase (ACCase) in algae were performed. Primers were designed to generate distinct size of amplicons depending on the ACCase allele(s) present within the algal cells using the fact that a (3`) mismatch does not prime in a PCR at a specific annealing temperature. Two primers were used:

Forward Primer:

ACVRG1R: 5`GCTGAGCCACCTCAATATATTAGAAACACC3`

Primer Reverse: **VRDIC+**: 5`GGACTAGGTGTGGAGAACC 3`

The PCR product indicating the presence of an ACCase allele with a (C) at nucleotide position 5341, yielded a 329-bp (base pair) fragment.

The thermocycler was programmed as the following :

* Pre-denaturation (one cycle):	94°C	5 min.
* Thirty five cycles:		
Denaturation	94°C	1 min.
Annealing	63°C	30 sec.
Extension	72°C	1 min.
* Final-extension (one cycle):	72°C	10 min.

Statistical analysis

Six replicates algae per treatment and three replicate measurements were carried out. All data were analyzed using SAS 9.2 and excel 2003 programs.

RESULTS AND DISCUSSION

Cell number

The results of the cell number growth in different concentrations during the six generations are presented in tables ١ and ٢. Data show that the cell number generally decreased with increasing herbicide concentrations especially with the highest two concentrations. However, in all herbicidal treatments, the cell number decreased in descending order with the advancement of generations, first, second and third generation due to herbicidal action. From the fourth generation the cell number increased in all treatments except with the highest two concentrations.

On the other hand, overall means of exposure periods, the cell number decreased in the first to the third generation, but from the fourth generation the cell number increased clearly with all exposure periods. These results suggest that the algae seek to increase the cell number as a mechanism of resistance to the herbicidal action in the last three generations.

Table (1): Effect of different concentrations (ppm) of clodinafop-propargyl herbicide on growth (Cell number *10^4) of green alga *Scenedesmus quadricauda* cultures during 1st to 3rd generations.

Time \ Treatment		Control	2X	1X	1/2X	1/4X	1/8X	1/16 X	Mean
T_0	1	1106	1103	1102	1102	1106	1102	1103	1103.42
	2	1409	597	601	638	750	788	802	797.86
	3	1623	436	456	510	572	659	717	710.43
T_{24}	1	1115	759	807	873	877	838	890	886.28
	2	1419	513	523	577	619	722	752	732.14
	3	1632	406	414	588	532	618	694	697.71
T_{48}	1	1121	683	699	810	844	853	871	840.14
	2	1428	483	488	521	593	700	695	701.14
	3	1656	389	392	466	507	591	675	668
T_{72}	1	1125	655	681	792	817	840	854	823.42
	2	1435	434	465	478	550	661	708	670.85
	3	1680	346	360	438	491	586	656	651
T_{96}	1	1131	619	655	770	800	826	836	805.29
	2	1447	425	434	452	505	623	627	644.71
	3	1712	335	339	427	464	575	613	637.86
Mean		1119.6	763.8	788.8	869.4	888.8	900.8	910.8	
		1427.6	490.4	502.2	533.2	603.4	698.8	709	
		1660.6	382.4	392.2	485.8	513.2	605.8	561.8	

Where 1= First generation 2 = Second generation 3 = Third generation
X = field dosage (168 ppm)

Free amino acid content

Free amino acid contents were determined after 0, 24, 48, 72 and 96 hrs from incubation with the different clodinafop-propargyl concentrations in the six generations. The results of the first to six generation are presented in tables 3 and 4. From the first to six generation data indicated that free amino

acid contents generally increased 24 hrs after exposure as a spontaneous reaction to the applied herbicide. From the first to third generation free amino acid content decreased after 48 to 96 hrs which might be attributed to decrease in cell number and herbicidal action.

Table (2): Effect of different concentrations (ppm) of clodinafop-propargyl herbicide on growth (Cell number *10^4) of green alga *Scenedesmus quadricauda* cultures during 4^{th} to 6^{th} generation.

Time \ Treatment		Control	2X	1X	1/2X	1/4X	1/8X	1/16 X	Mean
T_0	4	1106	338	347	661	678	701	802	661.86
	5	1409	267	271	608	877	863	1016	758.71
	6	1623	227	240	1310	1408	1622	1655	1162.14
T_{24}	4	1115	309	312	675	683	719	825	662.57
	5	1419	248	261	812	883	937	1023	797.57
	6	1632	223	232	1316	1416	1628	1690	1162.42
T_{48}	4	1121	294	296	696	694	739	836	666.57
	5	1428	238	254	819	894	977	1031	805.85
	6	1656	217	225	1322	1423	1434	1698	1167.8
T_{72}	4	1125	275	287	708	713	738	812	671.57
	5	1435	231	242	826	902	987	1406	812.71
	6	1680	213	220	1328	1428	1641	1731	1177.28
T_{96}	4	1131	263	272	715	725	745	857	673.71
	5	1447	223	234	833	909	993	1044	816.14
	6	1712	209	217	1334	1436	1581	1715	1194.84
Mean		1119.6	295.8	302.8	689	698.6	731.2	833.8	
		1427.6	241.4	252.4	779.6	893	951.4	1041.4	
		1660.6	217.8	226.8	1322	1422.2	1641.2	1709.8	

Where 4= Fourth generation 5 = Fifth generation 6 = Sixth generation
X = field dosage (168 ppm)

In the fourth and fifth generations free amino acid contents increased up to 48 hrs while decreased in the sixth generation, which apparently due to the recovery of the algae culture in the sublethal doses and emergence of a biotype resistant to the herbicide as well as increase in cell number. Free amino acid contents generally decreased after 72 to 96 hrs in the last three generations nearly to the normal level as a result of the occurrence of resistant population to the herbicide. These finding agree with that of Hartnett *et al.* (1987)

Allele-specific PCR

In addition to control (untreated culture), four samples from treated cultures (1/2X, 1/4X, 1/8X and 1/16X, field concentration) were subjected to the PCR reaction.

Table (3): Effect of different concentrations (ppm) of clodinafop-propargyl herbicide on free amino acid contents in green algae *Scenedesmus quadricauda* cultures during 1st to 3rd generations (± SD).

Time \ Treatment		Control	2X	1X	1/2X	1/4X	1/8X	1/16 X	Mean
T_0	4	0.184±0.003	0.201±0.001	0.174±0.002	0.185±0.001	0.181±0.002	0.182±0.003	0.185±0.003	0.184
	5	0.303±0.003	0.056±0.002	0.044±0.0020	0.247±0.004	0.265±0.003	0.285±0.002	0.294±0.005	0.213
	6	0.214±0.003	0.064±0.002	0.070±0.004	0.215±0.003	0.200±0.003	0.201±0.003	0.205±0.002	0.167
T_{24}	4	0.207±0.001	0.121±0.003	0.148±0.002	0.249±0.004	0.209±0.002	0.186±0.002	0.189±0.002	0.187
	5	0.305±0.003	0.068±0.003	0.068±0.002	0.278±0.002	0.278±0.003	0.288±0.002	0.285±0.003	0.224
	6	0.224±0.003	0.072±0.003	0.082±0.005	0.233±0.003	0.217±0.003	0.223±0.002	0.228±0.003	0.183
T_{48}	4	0.210±0.001	0.190±0.002	0.228±0.003	0.213±0.003	0.203±0.002	0.186±0.002	0.193±0.002	0.203
	5	0.304±0.002	0.276±0.002	0.284±0.002	0.287±0.002	0.285±0.003	0.293±0.002	0.294±0.002	0.289
	6	0.234±0.003	0.052±0.005	0.064±0.002	0.235±0.003	0.222±0.003	0.226±0.002	0.231±0.002	0.180
T_{72}	4	0.212±0.001	0.186±0.002	0.190±0.002	0.189±0.003	0.197±0.002	0.189±0.002	0.196±0.001	0.194
	5	0.307±0.002	0.083±0.002	0.093±0.002	0.291±0.003	0.297±0.001	0.298±0.002	0.299±0.001	0.238
	6	0.246±0.003	0.041±0.002	0.046±0.004	0.238±0.003	0.225±0.004	0.235±0.003	0.234±0.001	0.180
T_{96}	4	0.214±0.002	0.175±0.002	0.174±0.003	0.176±0.002	0.191±0.002	0.191±0.002	0.199±0.002	0.188
	5	0.313±0.002	0.085±0.003	0.094±0.001	0.298±0.003	0.298±0.003	0.301±0.002	0.303±0.002	0.241
	6	0.245±0.002	0.031±0.003	0.036±0.004	0.240±0.002	0.234±0.003	0.233±0.001	0.237±0.003	0.179
Mean		0.205	0.174	0.183	0.202	0.196	0.187	0.192	
		0.306	0.113	0.116	0.280	0.284	0.293	0.295	
		0.232	0.052	0.060	0.232	0.219	0.224	0.227	

	0.006		0.007		0.016
A (time)	0.023	B (concentration)	0.028	A*B	0.063
	0.001		0.001		0.003

Where 1 = First generation 2 = Second generation
3 = Third generation X = field dosage (168 ppm)

Table (4): Effect of different concentrations (ppm) of clodinafop-propargyl herbicide on free amino acid contents in green algae *Scenedesmus quadricauda* cultures during 4th to 6th generations (± SD).

Time \ Treatment		Control	2X	1X	1/2X	1/4X	1/8X	1/16 X	Mean
T_0	1	0.076 ± 0.006	0.077 ± 0.006	0.076 ± 0.006	0.076±0.006	0.077 ± 0.006	0.076 ± 0.006	0.076 ± 0.006	0.076
	2	0.206 ± 0.001	0.157 ± 0.001	0.191 ± 0.005	0.217±0.006	0.216 ± 0.001	0.223 ± 0.005	0.221 ± 0.001	0.204
	3	0.186 ± 0.002	0.140 ± 0.002	0.153 ± 0.002	0.172±0.001	0.181 ± 0.002	0.182 ± 0.003	0.185 ± 0.003	0.171
T_{24}	1	0.097 ± 0.001	0.642 ± 0.001	0.613 ± 0.002	0.602±0.001	0.585 ± 0.003	0.476 ± 0.004	0.476 ± 0.005	0.499
	2	0.209 ± 0.001	0.299 ± 0.002	0.245 ± 0.005	0.251±0.005	0.231 ± 0.005	0.232 ± 0.005	0.234 ± 0.005	0.256
	3	0.195 ± 0.003	0.168 ± 0.003	0.198 ± 0.002	0.174±0.002	0.174 ± 0.002	0.182 ± 0.001	0.197 ± 0.004	0.184
T_{48}	1	0.10 6± 0.005	0.334 ± 0.002	0.303 ± 0.002	0.316±0.002	0.284 ± 0.003	0.335 ± 0.002	0.314 ± 0.002	0.284
	2	0.212 ± 0.001	0.211 ± 0.002	0.220 ± 0.002	0.225±0.003	0.224 ± 0.003	0.206 ± 0.003	0.219 ± 0.002	0.217
	3	0.197 ± 0.003	0.17 ± 0.002	0.194 ± 0.001	0.177±0.003	0.184 ± 0.001	0.180 ± 0.002	0.194 ± 0.002	0.185
T_{72}	1	0.110 ± 0.001	0.136 ± 0.002	0.125±0.002	0.137±0.002	0.126 ± 0.002	0.138 ± 0.002	0.113 ± 0.001	0.126
	2	0.213 ± 0.005	0.194 ± 0.002	0.191±0.002	0.200±0.001	0.216 ± 0.002	0.200 ± 0.002	0.206 ± 0.002	0.203
	3	0.199 ± 0.002	0.170 ± 0.001	0.192±0.001	0.179±0.001	0.192 ± 0.001	0.176 ± 0.002	0.185 ± 0.003	0.185
T_{96}	1	0.112 ± 0.001	0.093 ± 0.004	0.091 ± 0.007	0.085±0.002	0.094 ± 0.001	0.101 ± 0.002	0.096 ± 0.001	0.096
	2	0.216 ± 0.001	0.155 ± 0.003	0.164 ± 0.003	0.184±0.002	0.195 ± 0.003	0.194 ± 0.001	0.193 ± 0.002	0.186
	3	0.203 ± 0.001	0.173 ± 0.001	0.189 ± 0.002	0.183±0.002	0.200 ± 0.002	0.175 ± 0.003	0.182 ± 0.001	0.186
Mean		0.100	0.256	0.241	0.243	0.233	0.225	0.215	
		0.211	0.221	0.202	0.215	0.216	0.211	0.214	
		0.196	0.164	0.185	0.177	0.186	0.179	0.188	

	0.002		0.002		0.005
A (time)	0.120	B (concentration)	0.142	A*B	0.318
	0.002		0.002		0.005

Where 4 = Fourth generation
6 = Sixth generation
5 = Fiveth generation
X = field dosage (168 ppm)

Primers ACVRG1R and VRDIC+ were designed to generate distinct size of amplicons depending on the ACCase allele(s) present within alga, where presence of nucleotide (C) or (T) instead of (A) at nucleotide position 5341 cause an isoleucine-leucine substitution in ACCase coding sequence that confers resistance to ACCase inhibitors Zagnitko *et al.* (2001) and Délye *et al.* (2002).

Amplification with primers ACVRG1R and VRDIC+ yielded a 329-bp fragment, indicating the presence of an ACCase allele with (C) at nucleotide position 5341. The PCR products were illustrated by agarose gel electrophoresis (figure 1). In this figure, the five samples, including control sample, showed band at size of 329 – bp indicating the presence of an ACCase allele with a (C) nucleotide. This result explain the resistance of these cultures to different treatments of herbicides.

In the present study only a small region of the algal ACCase gene has been examined and we cannot rule out the possibility of other mutations being involved in the production of insensitive enzyme. However, green algae *Scendesmus quadricauda* has been shown that the corresponding mutation is accompanied by other resistance mechanisms as free amino acids content and cell number change.

This mechanism, with the single isoleucine-leucine substitution can either reduce herbicide binding or affect its access to the target site. These results in agreement with Zhang and Devine (2000). Mutations in ACCase gene will enable molecular tools such as allele-specific PCR assay to monitor resistant alleles in the algal populations.

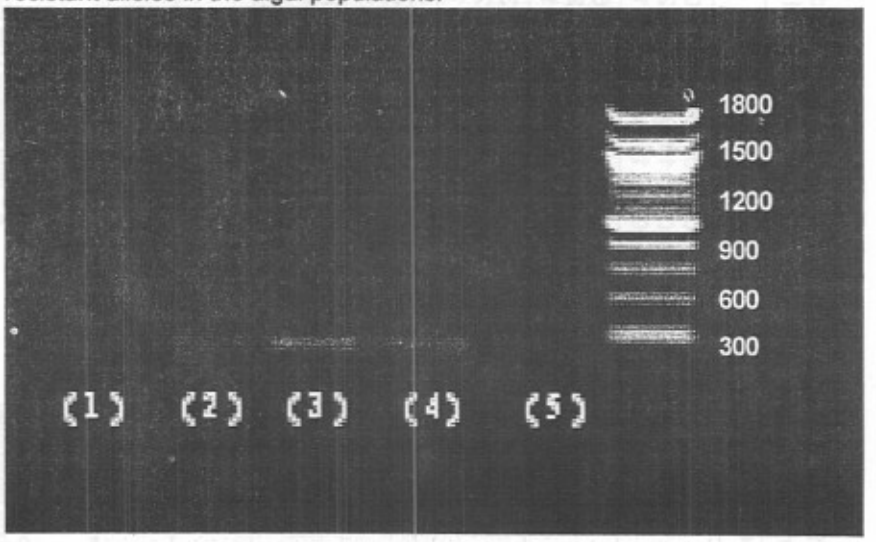

Fig (1): Allele-specific PCR analysis of five green algae *Scenedesmus quadricauda* treated samples by clodinafop-propargyl herbicide. Lane 1= control, Lanes 2 to 5= treated samples: 1/2X, 1/4X, 1/8X and 1/16X, where X = field concentration (168 ppm) using two primers ACVRG1R and VRDIC+.

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الأساس الجزيئى للمقاومة لمبيد الحشائش كلودينافوب بروبرجيل مثبط لأنزيم الأسيتيل كو انزيم أ كربوكسيليز فى الطحلب الأخضر سيندسمس كوادريكودا

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تمكن الطفرات فى جين (ACCase) من استخدام أدوات البيولوجيا الجزيئية مثل تقدير allele-specific PCR بغرض رصد السلالات المقاومة فى الطحلب الأخضر سيندسمس كوادريكودا *Scenedesmus quadricauda*. يحفز حدوث استبدال الأيزوليوسين-ليوسين فى الجين المشفر للبلاستيدات الخضراء (ACCase)المقاومة لمبيد الحشائش كلودينافوب بروبرجيل. عوملت مزرعة الطحلب الأخضر المذكور بتركيزات مختلفة للمبيد (٠، ١/١٦، ١/٨، ١/٤، ١/٢، ١ و ٢) من التركيز الحقلى . تم تقدير عدد الخلايا ومحتوى الأحماض الأمينية الحرة بعد (٠، ٢٤، ٤٨، ٧٢ و ٩٦) ساعة. فيما يتعلق بعدد الخلايا أظهرت النتائج أنه من الجيل الأول إلى الجيل الثالث حدث نقص فى عدد الخلايا خاصة فى أعلى تركيزين. ومن الجيل الرابع إلى الجيل السادس حدثت زيادة فى عدد الخلايا مع نقصان فى أعلى تركيزين. وفيما يتعلق بمحتوى الأحماض الأمينية أظهرت النتائج من الجيل الأول للسادس حدوث زيادة فى محتوى الأحماض الامينية بعد ٢٤ ساعة من المعاملة. ومن الجيل الأول للثالث حدث نقص فى محتوى الأحماض الأمينية بعد ٤٨ إلى ٩٦ ساعة. وفى الجيلين الرابع والخامس حدثت زيادة فى محتوى الأحماض الأمينية. وفى الجيل السادس حدث نقص فى الأحماض الأمينية، الأمر الذى قد يفسر باستعادة الطحلب لنشاطه فى الجرعات تحت القاتلة وظهور مجتمعات مقاومة للمبيد وأيضا لزيادة عدد الخلايا. هذا وتظهر نتائج allele-specific PCR وجود أليل(C) فى مزرعة الطحلب وهو ما قد يفسر مقاومته لمبيد الحشائش المستخدم.

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