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TOTAL PROTEIN ANALYSIS AND TRANSMISSION ELECTRON MICROSCOPY AS METHODS FOR DETECTION OF CLODINAFOP-PROPARGYL HERBICIDE RESISTANCE IN THE GREEN ALGA *Scenedesmus quadricauda*

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ABSTRACT

Green alga cultures of *Scenedesmus quadricauda* were treated with different concentrations of clodinafop-propargyl herbicide (0, 10.5 21, 42 and 84 ppm). Transmission electron microscopy and total protein analysis were applied to clarify the resistance of herbicide. Photos of longitudinal section in algal cell wall showed clear response to different concentrations of the herbicide. The response appeared as change in cell shape and increase in cell size, as well as thickness in the layers forming the cell wall which may indicate a type of resistance mechanism. Total protein analysis showed inducing or inhibition of the synthesis of some polypeptide fractions by herbicide treatments. These polypeptide fractions may have a role in resistance mechanism. These results encourage using green algal cell suspension of *S. quadricauda* for screening of herbicide resistance.

Keywords: Green alga, herbicide resistance, *Scenedesmus quadricauda,* total protein analysis, transmission electron microscopy, clodinafop-propargyl.

INTRODUCTION

Green algal cell suspensions are used in agrochemical research for screening herbicides (Ma *et al.,* 2002). 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).

From the previous studies, (Fedtke, 1991) and (Mohamed *et al.,* 2010) explained the observed resistance behavior in the green algae, *Chlamydomonas reinhardtii* and *Scenedesmus quadricauda* by the possible resistance mechanism of uptake, metabolism and lipid or amino acid composition. In addition to the role of metabolic detoxification in the resistance phenomenon and the importance of target site insensitivity, mutations in chloroplast genes such as acetyl-CoA carboxylase (ACCase) enables molecular tools such as allele-specific PCR assay to monitor resistance alleles in green algae (Chlorophyta) *S. quadricauda*. However, green alga *S. quadricauda* showed that the corresponding mutations is

accompanied by other resistance mechanisms such as free amino acids content and cell number change (Mohamed *et al.,* 2010).

The development of new cytological techniques, especially phase microscopy, cytochemical methods, and electron microscopy of thin sections has stimulated renewed investigations into the long controversial organization of blue-green algae (Ris and Singh, 1961). Susceptible and resistant cells were scanned and photographed using the scanning electron microscope to clarify the differences between resistant and susceptible cells (Ibrahim *et al.,* 2009).

In this work, methods of total protein analysis and transmission electron microscopy were used to detect and confirm resistance mechanisms to the herbicides in green alga (Chlorophyta) *S. quadricauda*.

MATERIALS AND METHODS

Culture conditions:

Green alga (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**). Alga was 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±2°C).

Herbicide treatments

Clodinafop, aryloxyphenoxypropionate (APP) a selective post-emergence herbicide, 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. Cells were exposed to the herbicide concentrations: control (0.0), 10.5, 21, 42 and 84 ppm. Samples for transmission electron microscopy and total protein analysis were taken after 6 generations of exposure to the concentrations of (0.0, 10.5, 21, 42 and 84 ppm).

Longitudinal section in alga cell wall

Samples (10.5, 21, 42 and 84 ppm) were taken in labeled test tubes and fixed in 5% glutheraldehyde for 24 hrs. The specimens were then washed in cacodylate buffer (0.1 M, pH 7.2) 3-4 times for 20 min. at each time and then post fixed in 1% osmium tetroxide for 2 hrs. After repeated washing in cacodylate buffer (4 X 20 min.), by using ascending grades of ethyl alcohol up to 100% (30, 50, 70, 80, 90 and 100% /2 hrs) dehydration was done using gelatin capsule embedded in Epon 812. For polymerization, the embedded samples were kept in an incubator at 35˚C for one day, at 45˚C for another day and three days at 60˚C.

From prepared blocks, using LKB ultra microtome, semi thin sections in thickness of 0.5-1µ were prepared. The sections were stained by toluidine blue, examined by light microscope and photographed. Regions for preparation of ultra thin sections were oriented by Leica ultramicrotome. The ultrathin sections in thickness of 500-800 Å were made and fixed on copper

grids (200µ meshes). The ultrathin sections were then contrasted in uranyl acetate for 15 min. and lead citrate for 5 min. and examined by a transmission electron microscope (Jeol, CX11) in Electron Microscope Unit, Assiut University, Egypt.

Protein extraction and purification

Five samples of algal cultures were used including one control sample (untreated culture) and four clodinafop-propargyl treated samples at concentrations corresponding to 10.5, 21, 42 and 84 ppm.

The protein was extracted using modified method of **Delye *et al.*, (2002)** as follows:

- Ten ml algal suspension were harvested by centrifugation at 5000 rpm for 5 min. then the supernatant was discarded.
- The pellets were ground to fine powder in liquid nitrogen and homogenized in 3 volumes (w/v) of extraction buffer (50 mM Tris-HCl, pH 8, 5 mM dithiothereitol, 1 mM EDTA).
- The samples were filtered through eight layers of filter paper and centrifuged at 5000 rpm for 15 min.
- The supernatant was brought to 80% saturation with solid ammonium sulfate ($(NH_4)_2\ SO_4$) and centrifuged at 5000 rpm for 15 min.
- The pellets were resuspended in a small volume of saturated $(NH_4)_2\ SO_4$ and stored at -20°C.
- The precipitated protein was centrifuged at 5000 rpm for 10 min. and dissolved in 2 volumes of phosphate-buffered saline buffer containing 5mM dithiothereitol then kept at 4°C.

Protein electrophoresis

Total protein analyses using SDS-PAGE according to Laemmli (1970) were used and protein molecular mass was determined using Gel-Pro Analyzer package (Media Cybernetica 1993-97). Control sample (untreated culture) and other four samples of treated cultures by different herbicide concentrations (10.5, 21, 42 and 84 ppm) were used in total protein analysis.

The proteins of the ACCase peaks were separated by SDS-PAGE on 4 to 15% gradient fast gel. Proteins were then transferred to Immobilon-P membranes using 10 mM CAPS (3-cyclohexylamine-1-propane sulfonic acid), pH 11 and 50 mM NaCl as the transfer buffer. Biotinylated proteins were probed using avidin-alkaline phosphatase (De Prado *et al.,* 2000).

RESULTS AND DISCUSSION

Transmission electron microscopy

Structure control of the cell wall of *S. quadricauda* consists of three layers: the inner cellulosic layer (CEL) which delimits individual cells; the outer pectic layer (PL) which binds the cells of the coenobium together; and a thin middle layer or trilaminar layer (TRL), bounded by membranes on either side. Comparison was made between conrtol cells of *S. quadricauda* and cells exposed to different clodinafop-propargyl concentrations (10.5, 21, 42 and 84 ppm).

Transmission electron micrographs of *S. quadricauda* control cell (Fig. 1) show that the cell wall consists of the three layers previously described. The outer pectic layer (PL) and the trilaminar middle layer (TRL) are thick, while

the inner cellulose layer (CEL) is thinness. Morphological analysis of algae in regulatory testing is known to be strongly recommended and could be extended beyond the *Scenedesmaceae*, as for example in *Chlamydomonas* the herbicide paraquat also caused morphological changes (Lurling, 2006) and (Hassanien, 2006) found significant increase in cell volume and wall thickness in ametyren selected biotype of *S. vacuolatus*.

At 10.5 ppm herbicide concentration (Fig. 2), the cell showed large thickness in the outer pectic layer (PL) and trilaminar layer (TRL), while the inner cellulosic layer (CEL) still thinness. In addition, change in shape and increase in the cell size was observed in all concentrations of herbicide treated cell, compared with control cell. At 21 ppm herbicide concentration (Fig. 3) the cell showed large thickness in the three layers especially in the inner cellulose layer (CEL) that was very thick which reflects the cell response to the herbicide dosage. This may suggest a type of resistance. At 42 ppm concentration (Fig. 4) the cell showed large thickness in the outer pectic layer (PL), while the middle layer trilaminar layer (TRL) and the inner cellulose layer (CEL) were thinness.

In (Fig. 5) at 84 ppm concentration, the cell showed very large thickness in all three layers, compared with control. In atrazine resistant *Conyza canadensis*, the thylakoid membrane lipids contained a lower amount of polar lipid and the fatty acid content exhibited a higher degree of unsaturation (Szigeti and Lehoczki, 2003). The high variability in sensitivity of different algal species to the same chemical substance can be explained by the morphology, cytology, physiology and genetics of the organisms (RojiEkova-Padrtova and MarGlek, 1999). The bioassay and biochemical data confirmed the role of herbicide metabolic degradation as a main mechanism of resistance toward herbicides. In addition, surface area of resistant cell was about four times greater than susceptible cell (Ibrahim *et al.,* 2009).

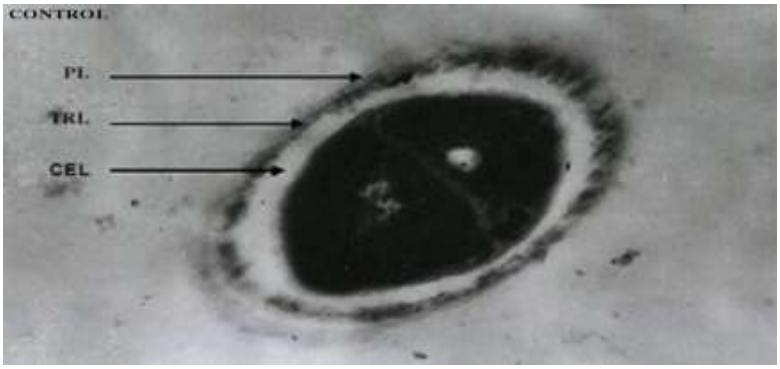

Fig. 1: Longitudinal section of control cell of *Scenedesmus quadricauda* after the sixth generation showing cellulosic layer (CEL), trilaminar layer (TRL) and pectic layer (PL). Magnification 14,000X.

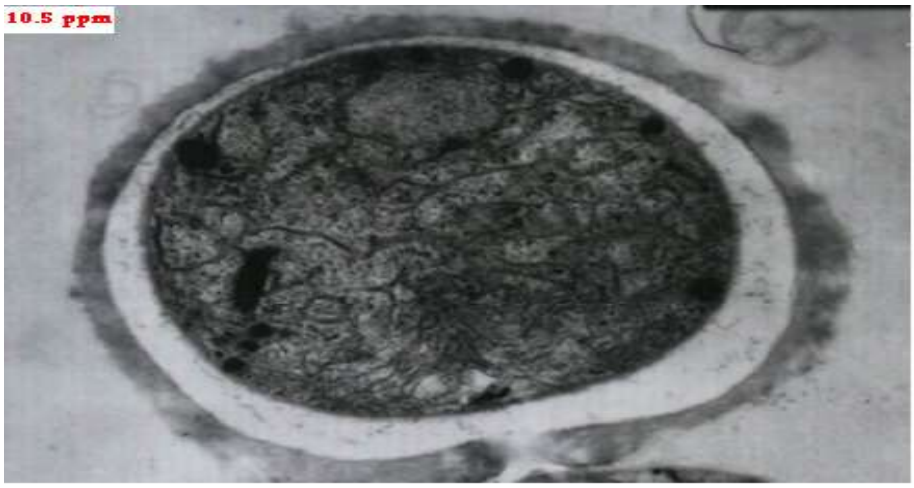

Fig. 2: Longitudinal section of *S. quadricauda* cell, after the sixth gneration of exposing to clodinafop-propargyl at 10.5 ppm. This photo shows cellulosic layer (CEL), trilaminar layer (TRL) and pectic layer (PL). Magnification 14,000X.

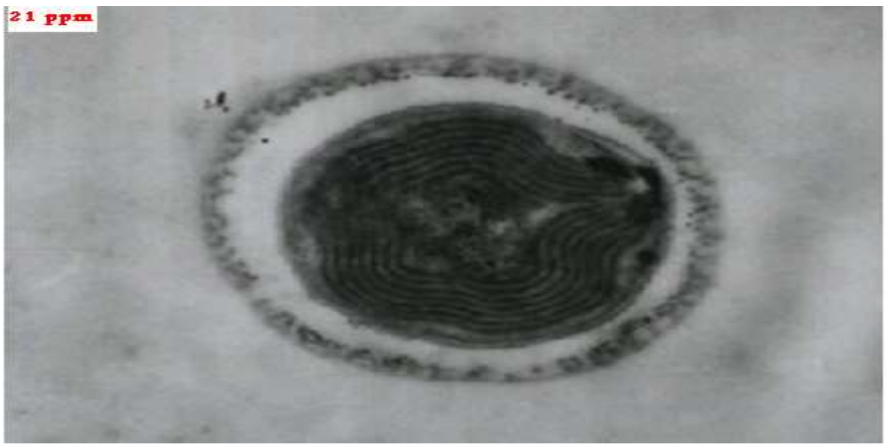

Fig. 3: Longitudinal section of *S. quadricauda* cell, after sixth generations of selection with clodinafop-propargyl at 21 ppm showing cellulosic layer (CEL), trilaminar layer (TRL) and pectic layer (PL). Magnification 14,000X.

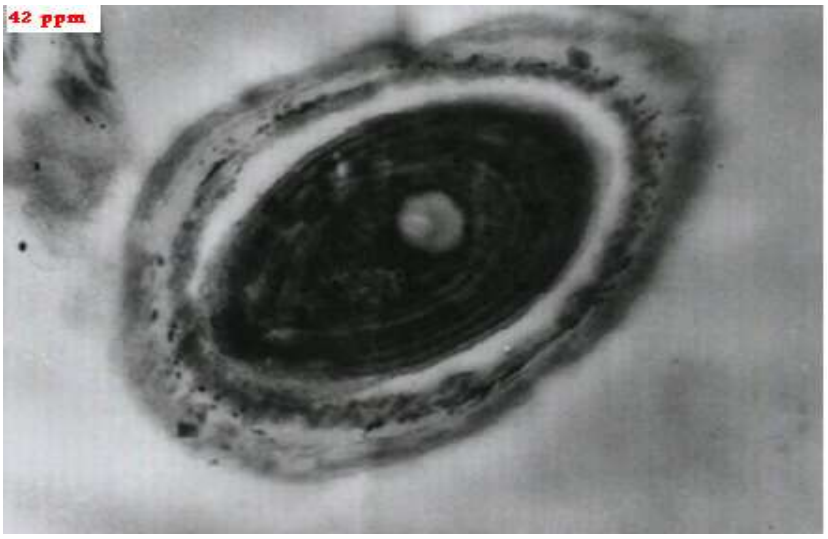

Fig. 4: Longitudinal section of *S. quadricauda* cell, after sixth generations of selection with clodinafop-propargyl at 42 ppm showing cellulosic layer (CEL), trilaminar layer (TRL) and pectic layer (PL). Magnification 14,000X.

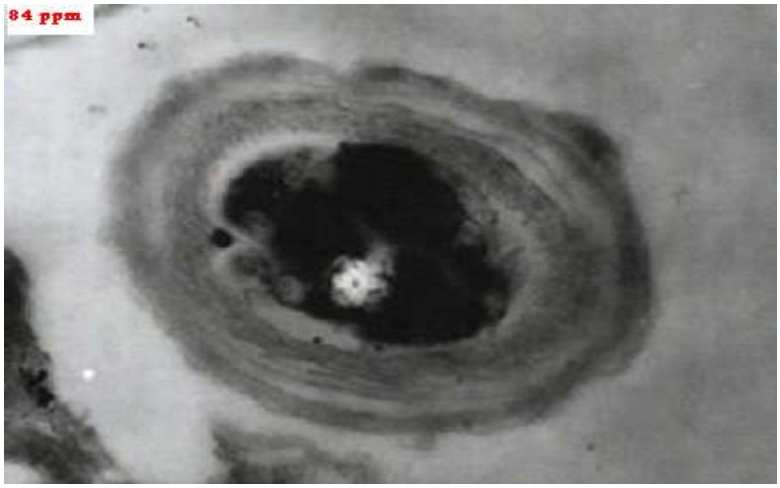

Fig. 5: Longitudinal section of *S. quadricauda* cell, after sixth generations of selection with clodinafop-propargyl at 84 ppm showing cellulosic layer (CEL), trilaminar layer (TRL) and pectic layer (PL). Magnification14,000X.

Total protein analysis by polyacrylamide gel electrophoresis (SDS-PAGE)

Total protein analysis was performed for five samples, i.e., control and four treated samples with 10.5, 21, 42 and 84 ppm herbicide concentrations. Protein
of purification fractions separated by SDS-PAGE (Fig. 6) and analysis of the molecular weight in Kda (Table 1) showed that the total number of protein fractions produced by all samples were 56 fragments. The number of protein fractions per sample varied between 10 to 12, with an average of eleven fractions per sample. These polypeptide fractions have a size ranged from about 9 to 110 Kda.

A total number of 56 fractions were shown with 40 polypeptide fractions being monomorphic (shared by all examined treated and untreated samples), and the other 16 polypeptide fractions were polymorphic (exist in some treated or untreated samples and not exist in the others). Polypeptide fractions with 72.17 and 19.81 kda were present only in control sample (untreated) and absent in all treated samples suggesting that the synthesis of these polypeptide may be inhibited by herbicide treatment. This result agreed with that found in algae and cyanobacteria by (Kotrikla *et al.,* 1999). However, the protein content in grains of greengram was found to be severely affected by the highest dose rates of atrazine and isoproturon, suggesting that the enzymes and other functional proteins are one of the target sites of herbicide toxicity, which subsequently leads to alteration in the protein metabolism of grains (Khan *et al.,* 2006).

On the other hand, the polypeptide fractions with about 45, 49, 50 and 14 Kda were found to be present only in the treated samples and absent in the untreated ones, suggesting that the herbicide treatments catalyze the synthesis of these polypeptides in the treated algal cells and may had a role in the resistance mechanism of algae against herbicide treatments.

These results are congruent with that reported by (Fayez and Kristen, 1996; Fayez, 2000) who reported modulated metabolic activities of the cell under stress conditions. Polypeptide fraction with 25.36 kda presented only in treated sample by 21 ppm herbicide concentration and absent in all other treated samples and the control. This result might be related with large thickness found in the inner cellulose layer (CEL) that treated by this herbicide concentration. Also, polypeptide fractions with 11.7 and 13.19 kda presented only in treated samples with 10.5 and 21 ppm herbicide concentration, respectively, and control sample but absent in the other treated ones. These results suggest that the synthesis of these polypeptides may be induced as a cell response to the herbicide treatments, as well as, it may explain the thickness in the three cell layers found in the herbicide treatments.

Using diuron herbicide on *Chlorella vulgaris* by (Fayez and Abd-Elfattah, 2007), protein and carbohydrate contents (total and soluble) of algae decreased significantly ($P<0.01$) with increasing herbicide doses. However calculation of the concentration of protein and carbohydrate on the basis of dry weight showed an increase in their contents with increasing herbicide

doses. The soluble protein content in control and at lowest dose (0.1µm) was 23 and 35 mg g/1 dry weight, respectively.

In our study, thickness in the layers forming the cell wall may suggest a type of resistance mechanism. In addition, herbicide treatment modulated metabolic activities of the cell as found in inducing or inhibiting synthesis of polypeptide fractions which may have a role in resistance mechanism. Finally, it is suggested that green algal cell suspensions of *S. quadricauda* could be used in agrochemical research for screening herbicide resistance.

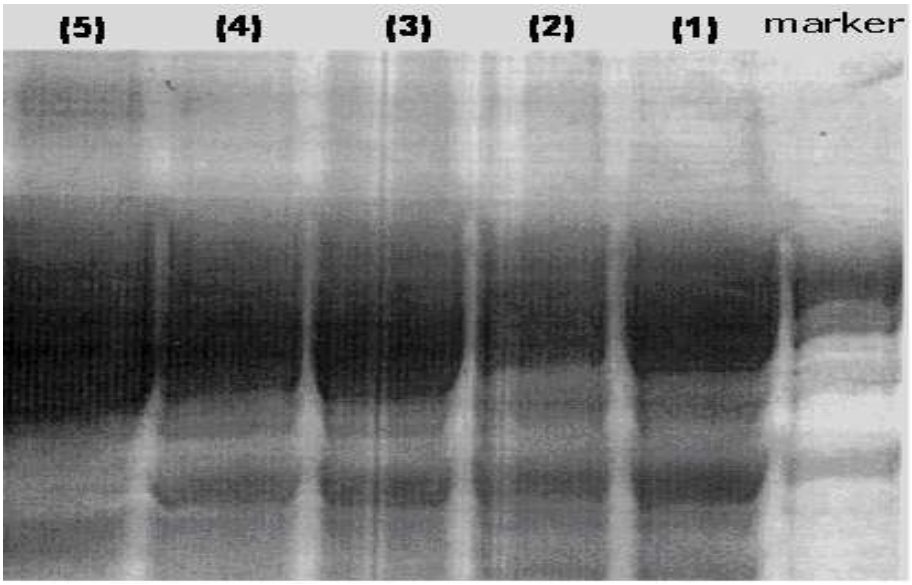

Fig. 6: Total protein of purification fractions separated by SDS-PAGE *Scenedesmus quadricauda* algal samples with different herbicide concentrations. From right: marker then samples 1= control, 2= 84 ppm, 3= 42 ppm, 4= 21 ppm and 5= 10.5ppm, respectively.

Table 1: Total protein fractions separated by SDS-PAGE and the molecular weight in Kda of *S. quadricauda* algal samples with different herbicide concentrations.

Lanes	Marker	Control	84 ppm	42 ppm	21 ppm	10.5 ppm
Protein fractions	Molecular weight in Kda					
r_1	200					
r_2	150					
r_3	120					
r_4		110.144	117.1403	112.5174	111.3494	118.933
r_5	100	91.224	94.0479	90.1709	96.9494	96.8888
r_6	85	86.601	84.1472	84.6427	85.1317	83.8803
r_7		72.174				
r_8	70	69.912	65.7467	68.5829	73.8018	70.0168
r_9	60					
r_{10}	50		45.4994	50.1025	45.9345	49.2109
r_{11}		31.264	27.4392	28.5599	35.7093	45.5937
r_{12}	30	29.648	26.2218	28.6184	28.0879	29.2133
r_{13}	25				25.3594	
r_{14}		22.828	21.367	21.378	21.7274	24.718
r_{15}	20	19.809				
r_{16}		16.18	16.028	17.995	16.4164	16.0126
r_{17}	15		14.01	14.0722	14.1657	14.26
r_{18}		11.024			13.194	11.729
r_{19}	10	9.144		10.912		

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تحليل البروتين الكلى وتقنية قطاعات الميكروسكوب الإلكترونى كطرق للكشف عن المقاومة لفعل مبيد الحشائش كلودينافوب- بروبرجيل فى الطحلب الأخضر سيندسمس كوادريكودا

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عوملت مزارع من الطحلب الأخضر سيندسمس كوادريكودا بتركيزات مختلفة من مبيد الحشائش كلودينافوب بروبرجيل (0، 10.5، 21، 42 و84 جزء فى المليون). استخدمت تقنية قطاعات الميكروسكوب الإلكترونى وتحليل البروتين الكلى للكشف عن المقاومة لفعل المبيد. أظهرت صور القطاعات الطولية فى جدار خلية الطحلب استجابة واضحة للخلية للتركيزات المختلفة من المبيد. وقد ظهرت الاستجابة فى صورة تغير فى شكل الخلية وزيادة فى حجمها وكذلك سُمك فى الطبقات المكونة لجدار الخلية، وهو الأمر الذى يدل على احتمال حدوث طراز من ميكانيكية المقاومة. أظهر تحليل البروتين الكلى حفز أو تثبيط تخليق بعض مكونات "عديد الببتيد" عند المعاملة بمبيد الحشائش. تشجع هذه النتائج على استخدام معلقات خلية الطحلب الأخضر سيندسمس كوادريكودا كأداة للتعرف على المقاومة لفعل المبيد.

كلمات مفتاحية: الطحالب الخضراء، المقاومة لفعل مبيدات الحشائش، سيندسمس كوادريكودا، تحليل البروتين الكلى، قطاعات الميكروسكوب الإلكترونى، كلودينافوب- بروبرجيل.

قام بتحكيم البحث

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