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INFLUENCE of SOME CLIMATE MODIFICATION TREATMENTS on GROWTH, BIOCHEMICAL and GENETIC VARIATION BASED on PROTEIN and ISOZYME MARKERS in (*Stevia rebaudiana*)

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ABSTRACT

Stevia rebaudiana, is an economically important medicinal plant act as sweetener substitute for diabetic and obese people. Climate modification causes a considerable influence on the production of several secondary metabolites of plants. In the present investigation, the effect of environmental factors on genetic divergence for three varieties of *Stevia rebaudiana* (Spanti; China and Eg1) by using differential response for different climate modification (black net, white net and plastic cover in comparing to without cover treatment (control) was studied. Data indicated that the application of different modification treatments showed that the use of nets had significant effect on air and substrate temperature and light intensity as well as relative humidity. In the same context, vegetative growth traits and biochemical analyses for stevioside and rebaudioside content had significant effects according to climate modification treatments. Electrophoresis banding patterns of total protein and isozyme (peroxidase) were obtained to characterize genetic relationship for both different treatments and genotypes. Based on the resolved soluble protein profiles it could be detected 11 and 13 polymorphic unique bands for different treatments and genotypes, respectively. The percentage of polymorphism for all studied treatment and varieties was 100 %. 12 and 13 polymorphic bands were detected in Isozyme marker (peroxidase) analysis for different treatments and genotypes, respectively. However, black net treatment and Spanti variety has the highest polymorphism percentage 100% by using cluster analysis of banding pattern for examined treatments and genotypes based on similarity index and UPGMA resulted four distinct clusters.

Key words: *Stevia rebaudiana*, stevioside, polymorphic bands, genotypes, peroxidase, isozyme, cluster analysis.

INTRODUCTION

Stevia rebaudiana is a small perennial herb belongs to the Asteraceae family; growing up to 65-80 cm tall, with sessile, oppositely arranged leaves. Different species of Stevia contain several potential sweetening compounds, with *S. rebaudiana* being the sweetest of all. Stevia is a semi-humid subtropical plant that can be grown easily like any other vegetable crop **(Yadav *et al.*, 2011).**

Stevia rebaudiana is one of the 154 members of the genus stevia, which produce sweet steviol glycosides **(Brandle *et al.*, 1998)**. Its plants were introduced to Egypt recently, and there are many trails to adapt this plant and growing it under Egyptian new reclamation areas. The leaves of Stevia are the source of diterpene glycosides, viz., stevioside and rebaudioside. Pure extract of stevioside is non-caloric which regenerated as a valuable natural sweetening agent because of its relatively

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good taste and chemical stability **(Ingle, 2008).**

The poor seed germination problem in this crop made a lot of obstacles towards large scale establishment of the crop and thereby making the available plant materials costly. Therefore, it is the best to propagate Stevia cuttings from a plant that has proven to be successful **(Goettemoeller and Ching, 1999)**. Cutting propagation is simply the excision of plant part (stem, root, or leaf cuttings) and nurturing the part to grow into a genetic replica of the original or parent plant. In addition, cutting enables researchers to clone selected genotypes and test the effects of various treatments among genetically identical individual. Thus, it can reduce the variation in intrinsic properties among individual plants **(Raji, 2012).** Stevia cultivation under protected cultivation condition is promising for increasing of the plant growth and avoiding the unsuitable weather conditions (temperature and extreme weather events). Plants grown in open fields of a semi-dry climate were subjected to direct sunlight, high temperatures and wind resulting in high crop evapotranspiration (ETc), therefore, demanding a large amount of water, while the roots of stevia cuts are not developed to supply water for plant during the acclimation stage. In contrast, shade-houses favor plant growth; since plants are less stressful, direct sunlight was avoided, temperature was lower, humidity was higher, wind speed reduced, and then lowers ETc.

Genetic identification of plants using proteins and isozymes electrophoreses considered an important method for plant identification and also to detect variations between varieties. Protein and isozymes specially are affected with physiological factors and surrounding environmental conditions. The major limitation of using biochemical fingerprints is the insufficient polymorphism among closely related varieties because proteins and enzymes are the products of gene expression; they may vary in different tissues, developmental stages and environments **(Sathisha *et al.*, 2012).** The identification of sunflower hybridsKBSH-1, TCSH-1, PKVSH-27, APSH-11 and DSH-1, their parental lines and the varieties , Morden ,Surya ,AKSF- 9, CO-2, CO-3, CO-4, GAUSF-15, NDSH-15 and SS-56was possible from the genotype specific intensity of dark, light and medium bands as well as their relative position in the seed protein and isozymes profiles analyzed by SDS-PAGE. Their characterizations were difficult through the total number of bands and as several of them were common in more than one genotype.

MATERIALS AND METHODS

Three *Stevia rebaudiana* varieties, *i.e.* Spanti, China and Eg1 were used in this study. Stevia cuts were provided by Sugar Crops Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt at March, 2016.

Cutting Preparation

Matured, young and healthy stem micro-cuttings were carried out using sharp scalpel, the length of cuttings ranged from 5 to 7 cm. The leaves attached to the cutting were reduced to three leaves. The stem was cut to include at least two nodes. Cut end was inserted/dipped immediately into indole butyric acid (IBA) with concentration of 1000 ppm described by **Abdullateef and Osman (2012)** and then cultivated in small polyethylene bags (0.35 liter). The polyethylene bags were filled with substrate mixture of peat moss: vermiculite: sand in the equal volume ratio (1:1:1).

Preparation of various climate treatments, the black polyethylene bags was put into four tables (1 m width ×1 m length) for the different varieties. The tables were supported by four wood legs with height of 0.5m. All varieties were represented equally (30 bags for each variety). The tables were covered by polyethylene sheet cover 200 µm, white net (30% shade), black net (60% shade) and

no shading table as shown in Fig. 1. The galvanized wires was used for support the different covers. The mist system was used in all treatments for irrigation cuttings.

Climate Factors Measurement

The measured climatic factors *i.e.* maximum and minimum air temperature °C (Air Temp.) as well as relative humidity (Ave. RH%) by using digital climatic sensors under all treatments during the experimental period (15 March till 15 May 2016). Digital thermo-hygrograph was used to measure temperature and relative humidity (model, SK-L200TH II). The maximum air temperature was recorded at 13:00; the average relative humidity was calculated by the average of maximum and minimum relative humidity every day; the average weekly maximum, minimum temperature and humidity has been calculated from the recorded daily climatic data. Maximum and minimum substrate temperature was measured by soil thermometer at five cm depth, and was recorded daily at mid-day during the experimental period. Also, Light intensity was measured in each treatment daily above the stevia plants at mid-day (13:00) by portable Lux-meter (Model FMC- 10M). The average weekly light intensity was calculated from the measured data.

Experiment Measurements

Vegetative characters

After 30 and 60 days from cultivation, five plants per treatment were taken to measure the following vegetative characters of each plot then the average per plant was calculated:

Number of leaves

By counting the leave allocated in stevia stems for five plant samples of each treatment.

Leaf area (cm^2) using LI-3000C portable area meter (standard technique No.5).

Plant height (cm)

By measuring the stem length from the soil surface to tip of the plant

Fresh and dry weight (g)

Five plant samples of each treatment. Five plants per treatment were taken to measure for fresh weight thin dried at 70°C for constant weight (16 -18 hr.).

Chlorophyll

The leaves number 4, 5 from the top of the plants were collected and total chlorophyll was determined according to SPAD501 Meter as SPAD units **(Monje and Bugbee, 1992).**

Stevioside and rebaudioside content

Stevioside and rebaudioside have been measured from fresh leaves taken from ten plants randomly after eight weeks from stevia cultivation with HPLC according to the method of (**Vanek *et al.*, 2001)**.

Protein and isozyme electrophoresis

Twelve *Stevia rebaudiana* accessions were used for fingerprinting study. After 60 days from cultivating stevia cuts, leaves were obtained from ten plants randomly for each accession so that SDS-protein and isozyme electrophoresis have been performed.

SDS-Protein electrophoresis

SDS-PAGE was performed on total (soluble and non-soluble) leaf protein fractions according to the method of **Laummli (1970)** as modified by (**Studier, 1973)**. One-dimensional SDS-PAGE was used to study proteins and separated based on their molecular sizes. Proteins migrated in the polyacrylamide gel toward the positive electrode. Altering the concentration of acrylamide changes the pore size of the gel. In this study, proteins were fractionated on 15% acrylamide concentration.

Isozyme electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was used to study different isozyme

variations. Peroxidase was identified by native-PAGE system (**Stegeman, 1980**).

Phylogenetic analysis

Phylogenetic relationship tree for *S. rebaudiana* was constructed using Hierarchical clustering of Past3 software based on UPGMA method.

Statistical analysis

Treatments were arranged in a factorial design with three replicates. The obtained data was subjected to statistical analysis of variance according to (**Snedecor and Cochran, 1980)**, and means of separation were done according to **Duncan (1955)** at the 5% level. Data analysis was performed by MSTATE Computer Statistically Analysis Program.

RESULTS AND DISCUSSION

Climatic Data

Average daily temperatures under different net colors, polyethylene sheet covered as well as opened field during cultivation of stevia cuts showed that the use of different climate modification treatments affected air temperature (Fig. 1). The highest temperature was recorded by polyethylene sheet cover treatment followed by control treatment (open field). Maximum temperature tended to be lower under the black and white net by 4-5^{o}C in comparison with open field due to the interception of radiation which is greater than the gain of temperature caused by the use of the nets due to their role in the interception of air circulation. This result is in agreement with previous finding by **Elad *et al.* (2007), Retamales *et al.* (2008)** and **Hashem (2011)** indicating that the influence of nets upon maximum temperatures under different net cover, they found that black net decreased the air temperature under greenhouse in comparison to open field and white net cover by 2-4^{o}C. Also data revealed that maximum temperature in all treatment had exceeded 29°C. Moreover, air temperature at sixth and seventh week increases under all tested treatments.

Average light intensity measured under each control treatment, black net, white net and polyethylene sheet cover through eight weeks shown in (Fig. 2). The highest light intensity during the eight weeks was found under the open field treatment (control) followed by white net. Similar results were reported by (**Abdrabbo *et al.*, 2013)**, who found a moderate increase in light intensity under open field comparing with white net. At the same period, light intensity under polyethylene sheet cover was lower than under the white net. Black net shading obtained the lowest values of light intensity comparing with other treatments, which agree with **Abdrabbo *et al.* (2013)** indicating lowest values of light intensity under black nets. **Stamps (2008)** reported that nettings, regardless of color, reduce radiation reaching crops underneath. Obviously, the higher the shade factor, the more radiation will be blocked.

Average relative humidity increased between 4-8% by the use of all covers compared to open field (Fig. 3). Data revealed that the polyethylene sheet cover treatment obtained the highest average relative humidity in comparison with the other treatments, which agreed with the results reported by **Hashem *et al.* (2011),** who indicated a 4-8% increase in humidity associated with the use of nets. Control treatment had the lowest relative humidity values during all tested weeks; it may be due to the decrease in evaporation in the open field, which agrees with **Elad *et al.*, (2007)** who reported a decrease in evaporation associated with the use of nets and a significant reduction in wind speed. Black net had a higher relative humidity values compered to white net by 2-3% during all weeks; may be due to the difference in air temperature under different nets that led to change in relative humidity. Results in Figs. 1 and 3 reflected a negative relationship between air temperature and relative humidity throughout the eight weeks. It is revealed that when temperature increases, relative humidity decreases. The highest value for relative humidity corresponds to the lowest temperature.

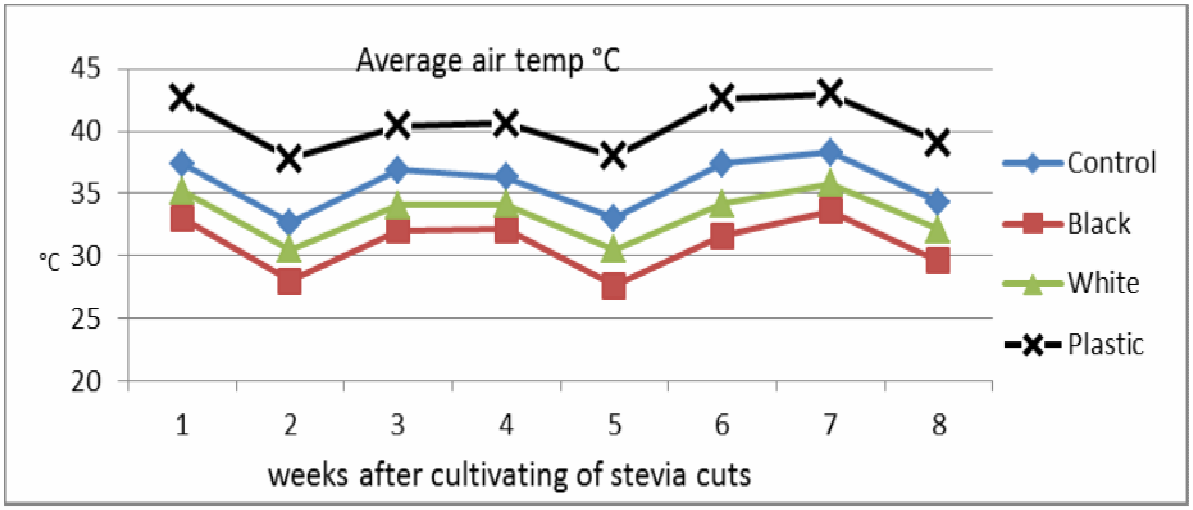

Fig. (1): The average air temperature under different treatments (control - black net-white net and Polyethylene cover) during 8 weeks

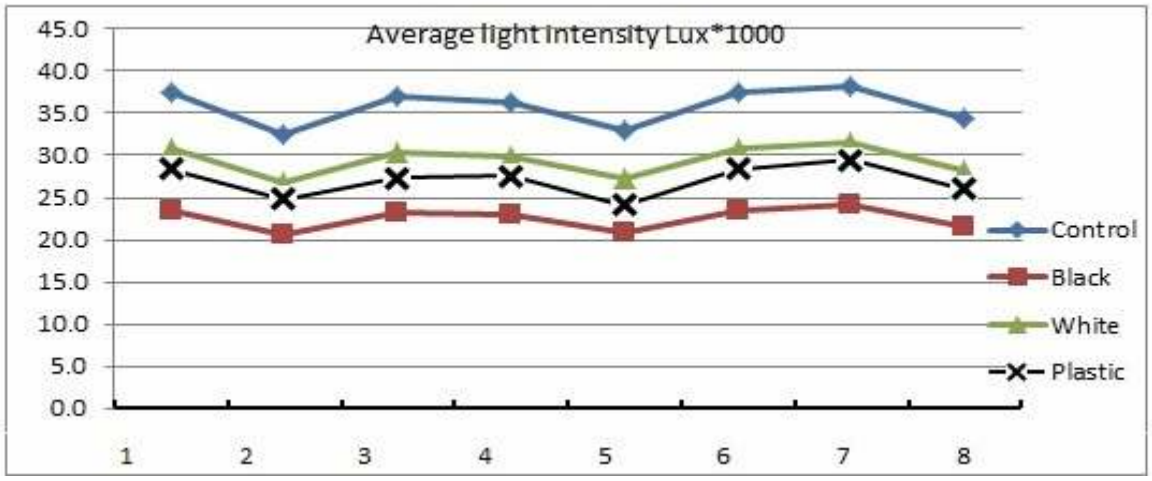

Fig. (2): The average light intensity by lux*1000 under different treatments (control - black net-white net and Polyethylene cover) during 8 weeks

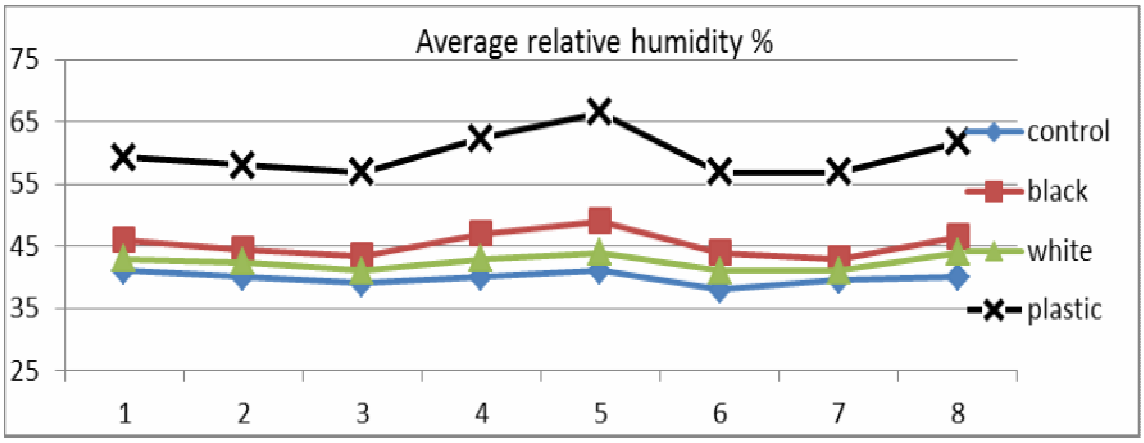

Fig. (3): The average relative humidity under different treatments (control - black net-white net and Polyethylene cover) during 8 weeks.

Vegetative Growth

The obtained results in Table 1 and 2 revealed that the cover treatments significantly affected the vegetative characteristics (number of leaves, leaf area, plant height, fresh and dry weights per plant and chlorophyll content) of Spanti, China and Eg1 varieties after 30 and 60 days from cultivating stevia cuts.

Concerning of vegetative growth, results in Tables 2 and 3 cleared that black net shading gave the highest mostly values for all studied traits; viz., number of leaves, leaf area/plant, plant height, fresh and dry weights per leaves as well as chlorophyll content with all studied varieties, followed by using of white net shading. Covering stevia with polyethylene plastic film came in the third order. Control treatment gave the lowest values for all studied traits. Similar results obtained by **Abdrabbo *et al.* (2013); Iglesias and Alegre (2006) and Al-Helal and Abdel-Ghany (2010)** who stated that vegetative growth of the plants under protection were bigger than those plants grow under open field conditions. It is clear; Spanti variety combined with black net gave the highest vegetative characteristics for stevia cuts after 60 days from cultivating followed by china variety combined with black net cover. Spanti variety combined with white net cover came in the third order. Eg1 combined with plastic cover or control treatment gave the lowest vegetative characters for stevia cuts after 60 days from cultivating of stevia cuts.

Results in Tables 1 and 2 revealed that there were significant differences for the interaction between climate modification treatments and varieties on all studied traits at all sampling dates. Generally it's obvious that all protection treatments used in this study affected vegetative characteristics; that's due to their influence against the injury of high temperature, relative humidity and light intensity (Fig. 4). The best treatment was happened with the plants shaded with black net followed by the use of white net; may be due to suitable environmental conditions (temperature, relative humidity and light intensity) prevailing during growing period. Similar results were reported by **Abdrabbo *et al.*, (2013),** indicating that increasing vegetative characteristics under white net cover could be attributed to the suitable climatic conditions. **Zakher and Abdrabbo (2014)** added that black net treatment led to a reduction in the temperature (2-3^{o}C) around plants and sun radiation. This result was disagree with **Hashem *et al.* (2011)** and **Abdrabbo *et al.* (2013)** they found that black net cover produced the lowest vegetative parameters on potato. High most temperature causes an increase in respiration, sometimes above the rate of photosynthesis which means that the products of photosynthesis are being used more rapidly than they are being produced. For growth to occur, photosynthesis must be greater than respiration. This result was in agreement with previous findings by **Fosket (1994)** and **Chuartzman *et al.* (2008)** they mentioned that reduce plant stresses improve the plants use of available resources. Results in Tables 1 and 2 also revealed that open field treatment gave the lowest vegetative characteristics which was agree with **Abdrabbo *et al.* (2013)** on potato and **Abul-Soud *et al.* (2014)** on brassica**.** Finally, Vegetative growth plant species is basically depend on the morphological, these features are changeable and sometimes difficult to observe, so it is necessary to be supported by molecular techniques. The SDS-PAGE study is one of the molecular marker as a tool to study molecular systematic for identification of genotypes based on proteins. These results will be presented in the third part.

Table (1): Effect of stevia cover treatments for number of leaves, leaf area and plant height after 30 and 60 days from cultivating stevia cuts

Treatment	Number of leaves / plant			Leaf area / plant (cm)			Plant height (cm)		
	Spanti	China	Eg1	Spanti	China	Eg1	Spanti	China	Eg1
After 30 days from cultivating stevia cuts									
Control	6.33_{f}	6.33_{f}	7.66_{f}	3.73_{f}	2.74_{g}	3.86f	3.60_{g}	3.16_{g}	3.03_{g}
Black net	12.00_{b}	11.00_{bc}	11.66_{bc}	6.67_{b}	6.90_{b}	8.40a	7.30_{a}	7.13_{ab}	6.70_{bc}
White net	15.00_{a}	15.33_{a}	10.33_{cd}	5.93_{c}	6.67_{b}	6.55b	6.36_{cd}	5.80_{d}	4.90_{ef}
Plastic	9.77_{de}	10.00_{de}	9.33_{e}	4.86_{e}	5.52_{d}	5.77cd	5.83_{d}	5.20_{e}	4.43_{f}
After 60 days from cultivating stevia cuts									
Control	16.67_{e}	13.67_{f}	14.00_{f}	2.06_{ab}	$1.80d_{e}$	1.76ef	13.00_{efg}	11.67_{g}	10.00_{h}
Black net	29.67_{a}	23.67_{bc}	24.33_{bc}	2.23_{a}	2.06_{ab}	1.86cd	25.00_{a}	25.33_{a}	21.00_{b}
White net	25.67_{b}	25.00_{b}	18.00_{e}	2.13_{ab}	2.10_{ab}	1.90cd	17.33_{c}	14.67_{de}	14.00_{def}
Plastic	22.33_{cd}	17.33_{e}	21.33_{d}	2.20_{ab}	1.96_{ab}	1.66f	15.00_{d}	13.67_{def}	12.67_{fg}

Means have the same letter(s) did not significantly differ at 0.05 level of probability according to Duncan's Multiple Range Test.

Table (2): Effect of stevia cover treatments on Fresh and dry weight of stevia leaves and chlorophyll content after 30 and 60 days from cultivating stevia cuts.

Treatment	Fresh weight (g)			Dry weight (g)			Chlorophyll content (SPAD) unit		
	Spanti	China	Eg1	Spanti	China	Eg1	Spanti	China	Eg1
After 30 days from cultivating stevia cuts									
Control	5.803_{d}	5.177_{f}	5.320_{f}	1.497c	1.370e	1.560b	33.23_{f}	34.27_{e}	31.87_{h}
Black net	5.537e	4.123h	4.600g	1.700a	1.283f	1.440d	36.57_{a}	36.10_{b}	35.77_{c}
White net	3.377 j	2.897 k	3.587i	1.133g	0.980h	1.357e	35.56_{c}	35.50_{c}	34.70_{d}
Plastic	6.763a	6.107c	6.520b	0.790j	0.807ij	0.853i	32.47_{g}	31.90_{h}	33.23_{f}
After 60 days from cultivating stevia cuts									
Control	10.92a	9.963b	9.903b	2.833a	2.720a	2.787a	32.12_{bc}	34.83_{a}	29.79_{e}
Black net	8.863c	6.580e	7.670d	2.647a	1.993bc	2.273b	32.94_{b}	35.22_{a}	34.99_{a}
White net	5.627f	4.833g	5.980f	1.803cd	1.520de	2.070bc	34.87_{a}	35.24_{a}	34.70_{a}
Plastic	6.857e	5.490f	6.637e	1.457e	1.420e	1.860c	24.57_{f}	30.55_{de}	31.50_{cd}

Means have the same letter(s) did not significantly differ at 0.05 level of probability according to Duncan's Multiple Range Test.

Stevioside and Rebaudioside Content

Results presented in Table 3 show significant differences for the interaction between climate modification treatments and varieties on stevioside and rebaudioside content of stevia plants. Regarding stevioside content, *Spanti* variety gave the highest values with all treatments in both fresh and dry weight followed by Eg1 variety. China variety came in third order. White net shading treatment combined with Spanti variety gave the highest values while plastic cover treatment combined with china variety gave the lowest values in fresh and dry weight. In the other side, rebaudioside content achieved the highest values with Eg1 variety followed by china variety. Spanti variety came in the third order. Control treatment combined with China variety gave the highest values in both fresh and dry weight, although black net shading combined with Spanti variety gave the lowest values.

Protein and Isozyme Electrophoresis under Microclimate Conditions

Assessment of genetic diversity through SDS-PAGE is easy and cheap. Protein markers have emerged as a possible tool in studies on genetic variability and have effectively been employed for identification of varieties in a number of crop plants. In addition, Isozyme analysis offers more reliable mean for producing genetic profiles and elucidation of genetic relationships within different varieties. So, the purpose of this part was to study the effect of environmental factors on the genetic diversity for the studied varieties of *Stevia rebaudiana* using different modified climate on molecular basis.

The present investigation of SDS denatured proteins showed differences in a number of bands, bandwidth. Unevenness in intensity was noticed in many protein bands that exhibited the quantity of protein peptides swelling up at a specific molecular weight. **Aparadh *et al.* (2012)** compared the banding pattern of seeds and leaf proteins of cleome species by SDS page. Protein profiles and Peroxidase isozyme profiles of stevia for (four treatments/three genotypes) are illustrated in Fig. 5. Bands were observed having molecular weights ranged from 5 to 50 kDa. Meanwhile, Fig. 6 Show the pattern of Isozyme (peroxidase) markers for studied climate modification treatments.

Table 4 showed the summary of polymorphisms and numbers of bands pattern resulted from soluble proteins and peroxidase marker for studied climate modification treatments. From one hand, for soluble proteins we can notice that among about 14 bands could be detected. 13 of them were polymorphic bands and 11 were unique bands. It was clear that there is no any monomorphic band in all the treatments except control treatment. In contrast, there were 6 unique and polymorphic bands for white net treatment, 3 unique and polymorphic bands for polyethylene cover treatment and 3 polymorphic bands two from them were unique bands for control treatment. The percentage of polymorphism for all cover treatments were 100% while the percentage of polymorphism for control treatment was 75%. This means that there is less variability among evaluated genotypes under this treatment comparing with other treatments. From other hand, in isozyme analysis 12 Polymorphic bands were detected from total 18 bands. They are (1, 2 and 2) monomorphic band were observed in white net treatment & control and polyethylene cover treatments respectively. In contrast, there is no monomorphic band in black net treatment. In the same time, black net treatment gave highest percentage of polymorphism 100% of total bands comparing to white net treatment (80%) control treatment (60%) and polyethylene cover treatment (50%) polymorphism. Moreover, black net treatment was having 3 unique bands and it was the highest treatment followed by polyethylene cover treatment that has 2 unique bands.

Table (3): Fresh and dry weight stevia leaves on stevioside and rebaudioside content.

Treatments	Stevioside content (%)			Rebaudioside content (%)		
	Spanti	China	Eg1	Spanti	China	Eg1
			In fresh weight			
Control	0.63_{c}	0.45_{g}	0.51_{f}	0.53_{i}	1.87_{a}	0.98_{d}
Black net	0.40_{h}	0.41_{h}	0.41_{h}	0.36_{l}	0.94_{f}	0.97_{e}
White net	0.74_{a}	0.36_{i}	0.65_{b}	0.51_{j}	1.06_{c}	1.08_{b}
Plastic	0.54_{e}	0.33_{j}	0.60_{d}	0.48_{k}	0.60_{h}	0.84_{g}
			In dry weight			
Control	1.98_{d}	1.30_{h}	1.45_{g}	1.65_{g}	6.04_{a}	2.79_{e}
Black net	1.55_{e}	1.53_{ef}	1.47_{g}	1.38_{h}	3.46_{c}	3.46_{c}
White net	1.55_{e}	1.21_{i}	2.16_{c}	1.68_{g}	3.54_{bc}	3.60_{b}
Plastic	2.46_{b}	1.26_{hi}	2.14_{c}	2.28_{f}	2.29_{f}	3.00_{d}

Means have the same letter(s) did not significantly differ at 0.05 level of probability according to Duncan's Multiple Range Test.

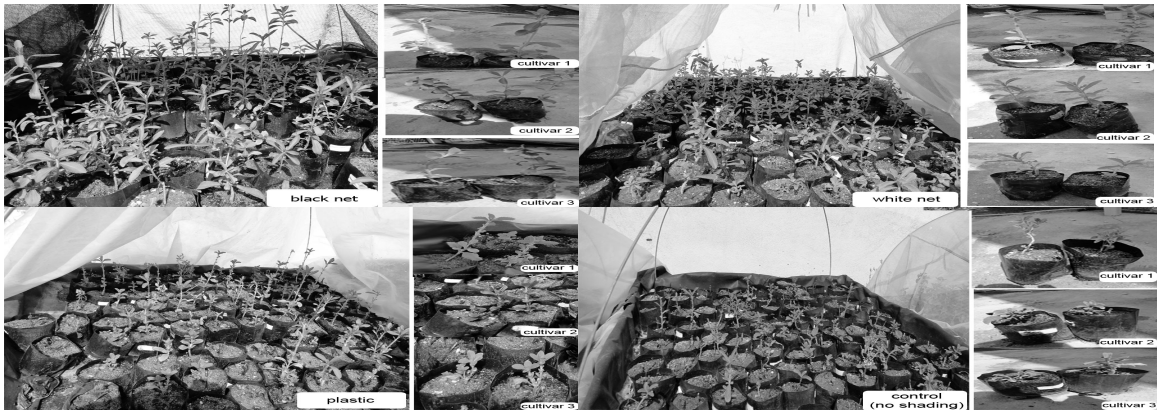

Fig. (4): Difference between three variety s of stevia under different treatments (black net, white net, plastic and no shading) after 60 days from cultivating.

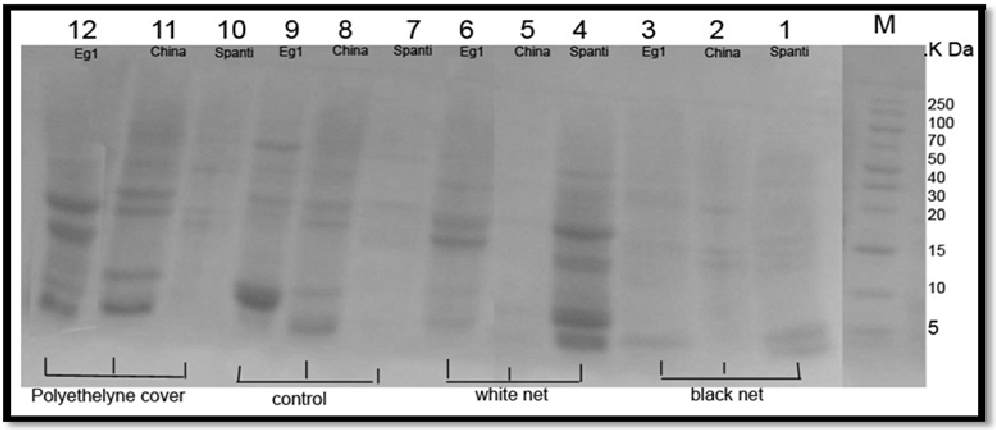

Fig. (5): Soluble protein profiles in twelve samples of stevia on SDS polyacrylamide gel.

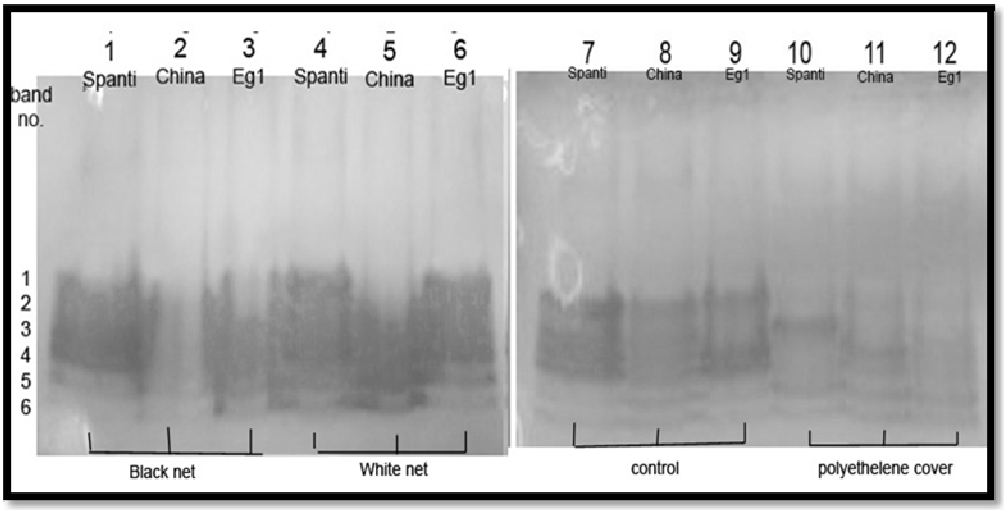

Fig. (6): Peroxidase isozyme profiles in twelve samples of stevia on SDS polyacrylamide gel.

Table (4): Polymorphisms of bands pattern resulted from different molecular systems electrophoresis for four treatment of stevia.

	Treatment	Total bands	Monomorphic bands	Polymorphic bands	Unique bands	% of polymorphism
Soluble proteins	black net	1	0	1	0	100 %
	white net	6	0	6	6	100 %
	control	4	1	3	2	75 %
	plastic cover	3	0	3	3	100 %
	Total	14	1	13	11	93 %
Peroxidase	black net	4	0	4	3	100 %
	white net	5	1	4	1	80 %
	control	5	2	3	0	60 %
	plastic cover	4	2	2	2	50 %
	Total	18	5	13	6	67 %

Unique bands in the SDS-PAGE analysis enabled the studied treatments genotypes to be distinguished from each other. The origin of these protein bands may be attributed to mutations at the priming site. By far, these bands are used also for the identification of the various genotypes (**Bakry, 2005**). In fact, unique bands are very important due to the presence of the certain unique sequences of protein in only one genotype which are considered as "labeling" characteristic to ensure distinction in every genotype. The biological functions of proteins include roles in metabolism, photosynthesis, transport, response to abiotic stimulus and response to stress (**Bibi *et al.*, 2009**). The appearance and disappearance bands showed in different climate treatment depending upon the gene over and under expression depending on the demand at that treatment.

In addition, Table 5 showed summary for polymorphisms and numbers of bands pattern resulted from soluble proteins and peroxidase marker for studied genotypes. The observations of this part showed a large variation in the number of protein bands among the studied genotypes with 100% polymorphism percentage.

Table (5): Polymorphisms bands pattern resulted from different molecular systems electrophoresis for three genotypes of stevia.

	Treatment	Total bands	Monomorphic bands	Polymorphic bands	Unique bands	% of polymorphism
Soluble proteins	**Spanti**	3	0	3	2	100%
	China	3	0	3	1	100%
	Eg1	7	0	7	5	100%
	Total	13	0	13	8	100%
Peroxidase	**Spanti**	6	0	6	2	100%
	China	5	1	4	3	80%
	Eg1	4	1	3	0	75%
	Total	15	2	13	5	80%

From one hand, there are a total of 13 soluble proteins polymorphic bands and 8 of them were unique bands. However, Eg1 genotype recorded highest number of polymorphic bands (7). Five of them were unique bands at the molecular weights; 50, 40, 30, 15 and 5 KDa as a marker bands for this genotype. Moreover, Spanti genotype has three polymorphic bands two of them were unique bands at the molecular weight 40 and 20 KDa which may be used as markers for this genotype. One unique band from three polymorphic bands at molecular weight of 5 KDa were found in the China genotype. The polymorphism observed was considered to be reasonably high. These proteins may be much important in crop improvement programs through breeding and genetic engineering. Similar results were obtained by **Odeigah *et al.* (1999)** in varieties of *C. annuum L.* and *C. frutescens L.*

From other hand, in isozyme analysis 13 polymorphic bands were detected from total 15 bands. A total of six bands are scored for the Spanti genotype. Results revealed that Spanti genotype have the highest polymorphism percentage 100% from all the studied varieties and two bands were characterized in the Spanti genotypes as a marker bands. In this regard, China genotype percent of polymorphism was 80% with three unique bands from four polymorphic bands for china variety. Moreover, China and Eg1 have one monomorphic band. Generally, the genotypes showed considerable variation in isozyme polymorphic band number ranged from 3-6 bands in agreement with (**El-Fiky *et al*., 2002; Bekhet *et al*., 2008; Colic *et al*., 2010; El-Bakry *et al*., 2014**) who found that banding patterns of peroxidase revealed high levels of polymorphism among different genotypes.

Phylogenetic analysis

Genetic relationship between different treatments and genotypes was studied by using cluster analysis of banding pattern for examined treatments and genotypes based on similarity index and UPGMA resulted four distinct clusters (Fig.7).

Proteins clusters analysis

Fig. 7 (A, B&C) showed three classified dendrograms for genotypes, treatments and the interaction between them. The first dendrogram contained two major clusters; one of them included Spanti variety, while

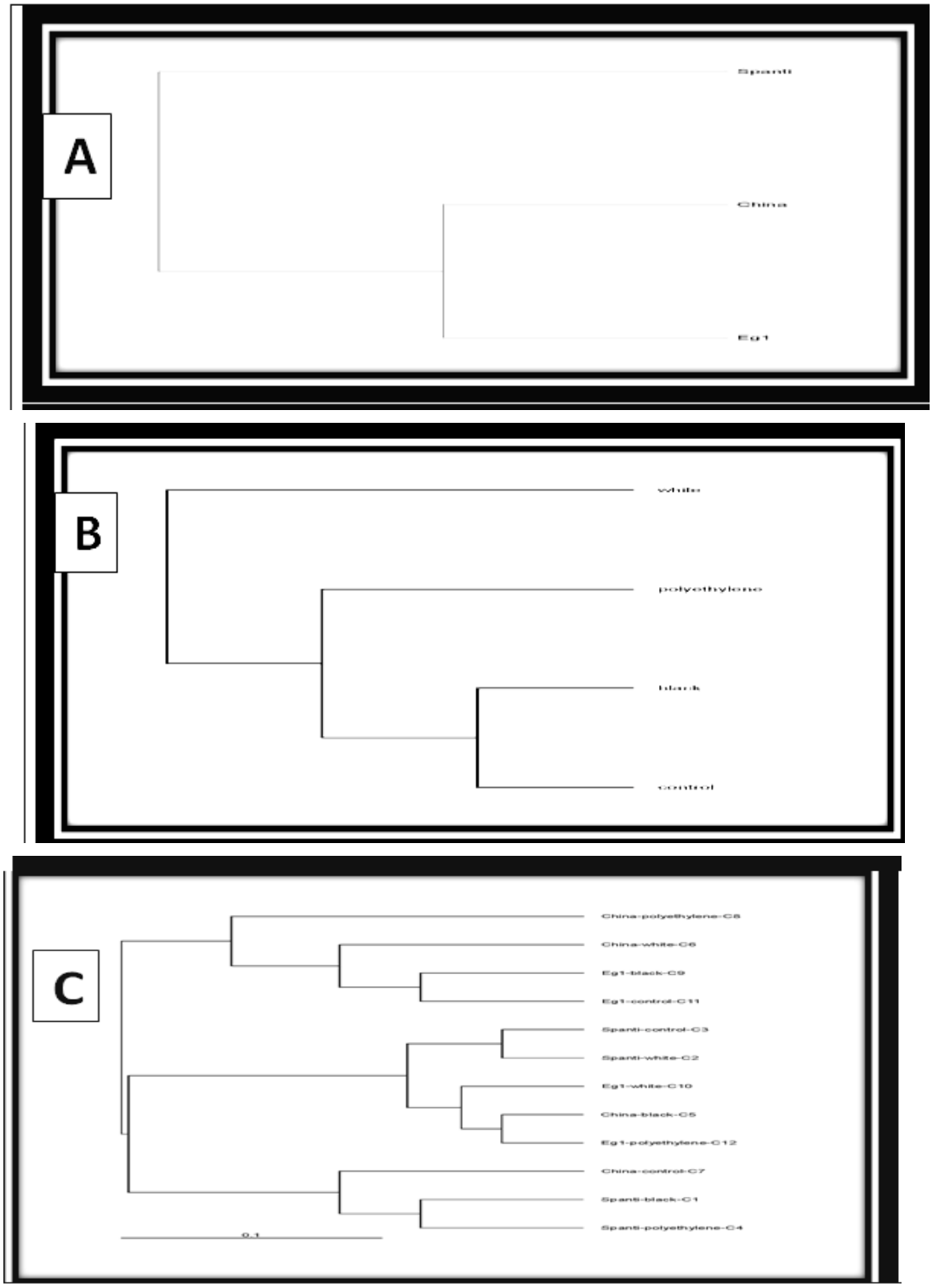

Fig. (7): Dendrogram presenting the association among different (A) varieties (B) *treatments (C) Treatments * varieties / Proteins and isozyme clusters analysis.

the other one included China and Eg1 varieties. While the second dendrogram contained three major clusters, white net represented one clade, then control and black net treatment in the other cluster. The third dendrogram contained two major clusters; the first of them contained four clades, while the second cluster contained two sub clusters of 5 and 3 clades, respectively. As a general view, these results revealed considerable amount of variability observed among the three studied genotypes. It is obvious that all treatments and genotypes clearly differed in their relationship in SDS-page polymorphic bands and unique bands. Occurrence of such protein polymorphism is a reflection of the complex genetic nature of the genotypes. Polymorphism in the present work proves the above said scientific fact. Moreover, this cluster is divided for more sub cluster and sub- sub clusters according for presence or absence bands. It is worth mentioning that there are two different sub-sub clusters that include two different treatment/genotypes that have only one unique band. The dendrogram revealed high genetic diversity because most of the treatments/genotypes are grouped in different cluster.

Conclusion

Using of climate modification treatments is believed to be a reliable technique for propagation of stevia cuttings. All of the treatments in this research were contributed to the development of shoot cuttings of stevia. Based on results obtained; there were significant differences between genotypes and treatments in parameters studied growth performance of stevia shoot cuttings was improved by application of different cover treatments. Besides, black net (60% shade) and white net (30% shade) was reliable to increase vegetative growth and biochemical content *i.e.* stevioside and rebaudioside in stevia plant.

In the current investigation our results suggest that white net treatment with Eg1 and black net treatment with Spanti variety showed better marked protein profiles with reference to their band presence or absence, intensity and molecular weights can be recommended in breeding programs to develop *Stevia rebaudiana* varieties. It is recommended to perform further analysis using DNA molecular markers that will help to understand deeply the genetic variability of the studied genotypes. So, the results achieved in this study can be useful for upcoming research projects in order to achieve mass production of stevia.

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REFERENCES

Abdrabbo, M.A.; Farag, A.A. and Abul-Soud, M. (2013). Intercropping effect on potato under net house as adaption procedure of climate change impacts. Res., 5 (6): 48-60.

Abdullateef, R.A. and Osman, M. (2012). Effects of stem cutting types, position and hormonal factors on rooting in *Stevia rebaudiana* Bertoni. J. Agric. Sci., 4: 49-57.

Abul-Soud, M.A.; Emam, M.S.A. and Abdrabbo, M.A.A. (2014). Intercropping of some brassica crops with mango trees under different net house color. Res. J. Agric. and Bio. Sci., 10 (1): 70-79.

Al-Helal, I.M. and Abdel-Ghany, A.M. (2010). Responses of plastic shading nets to global and diffuse PAR transfer: Optical properties and evaluation. Wageningen J. Life Sci., 57: 125-132.

Aparadh, V.T.; Amol, V.P. and Karadge, B.A. (2012). Comparative analysis of seed and leaf proteins by SDS PAGE Gel electrophoresis within Cleome species. Int. J. Advance Life Sci., 3: 50-58.

Bakry, A.M.S. (2005). Identification studies on Egyptian cowpea by fingerprinting technique. Ph. D. Thesis, Fac. Agric., Al-Azhar Univ., Egypt.

Bekhet, M.M.M.; El-Shawaf, I.I.; Hassan, A.M.; El-Saied, F.M. and Masoud, I.M. (2008). Genetic distances between three Ushaar (*Calotropisprocera* (ait) F.) genotypes as measured by RAPD and ISSR techniques. 4th Conf. Sustainable Agric. Dev. Fayoum Univ., Egypt, 79-90.

Brandle, J.E.; Starratt, A.N. and Gijzen, M. (1998). *Stevia rebaudiana*: Its agricultural, biological, and chemical properties. Can. J. Plant Sci., 78 : 527-536.

Bibi, N.; Hameed, A.; Ali, H.; Iqbal, N.; Haq, M.A.; Atta, B.M.; Shah, T.M. and Alam, S.S. (2009). Water stress induced variations in protein profiles of germinating cotyledons from seedlings Oofchickpea genotypes. Pak. J. Bot., 41 (2): 731-736.

Chuartzman, S.G.; Nevo, R.; Shimoni, E.; Charuvi, D.; Kiss, V.; Ohad, I.; Brumfeld, V. and Reich, Z. (2008). Thylakoid membrane remodeling during state transitions in Arabidopsis. Plant Cell, 20 : 1029-1039.

Colic, S., Milatovic, D.; Nikolic, D. and Zec, G. (2010). Isoenzyme polymorphism of almond genotypes selected in the region of northern Serbia. Hort. Sci., 37 (2): 56–61.

Duncan, D.B. (1955). Multiple Range and Multiple F-tests. Biometrics, 11: 1-42.

Elad, Y.; Messika, Y.; Brand, M.; David, D.R. and Sztejnberg, A. (2007). Effect of colored shade nets on pepper powdery mildew (Leveillulataurica). Phytoparasitica, 35:285-299.

El-Bakry, A.A.; Hammad, I.A. and Rafat, F.A. (2014). Polymorphism in Calotropisprocera: preliminary genetic variation in plants from different phytogeographical regions of Egypt. Rendiconti Lincei., 25 (4): 471-477.

El-Fiky, Z.A.; Hussein, M.H.; Mohamed, E.M. and Hussein, H.A. (2002). Biochemical and molecular genetic studies using SDS-protein, isozymes and RAPD-PCR in some common bean (*Phaseolus vulgaris* L.) varieties. Arab J. Biotech., 5 (2): 249-262.

Al-Helal, I.M.; Abdel-Ghany, A.M. and Fosket, D.E. (1994). Plant Growth and Development: A Molecular Approach. Academic Press, New York, p. 580.

Goettemoeller, J. and Ching, A. (1999). Seed germination in *Stevia rebaudiana.* Perspectives on new crops and new uses. ASHS Press. Alex., Va.

Hashem, F.A.; Medany, M.A.; Abd El-Moniem, E.M. and Abdallah, M.M.F. (2011). Influence of green-house cover on potential evapotranspiration and cucumber water requirements. Annals of Agric. Sci., 56:49-55.

Iglesias, I. and Alegre, S. (2006). The effect of anti-hail nets on fruit protection, radiation, temperature, quality and profitability of 'Mondial Gala' apples. J. Appl. Hort., 8 (2): 91-100.

Ingle, M.R. (2008). Effect of growth regulators and environments on rooting of Stevia cuttings (*Stevia rebaudiana* Bertoni). M.S. Thesis, Univ. Agric. Sci., Dharwad, India.

Laummli, U.K. (1970). Cleavage of structural head of bacteriophage. Nature, 722: 680-685.

Monje, O.A. and Bugbee, B. (1992). Inherent limitations of nondestructive chlorophyll meters: a comparison of two types of meters. Hort. Sci., 27: 69-71.

Odeigah, P.G.; Oboh, C.B. and Aghalokpe, I.O. (1999). The characterization of Nigeria varieties of pepper, Capsicum annum and Capsicum frutescens by SDS-polyacrylamide gel electrophoresis of seed protein. Genet. Res. and Crop Evolution, 46: 127-131.

Raji, A.A. (2012). Effects of stem cutting types, position and hormonal factors on rooting in *Stevia rebaudiana*Bertoni. J. Agric. Sci., 4 (1): 49-57.

Retamales, J.B.; Montecino, J.M.; Lobos, G.A. and Rojas, L.A. (2008). Colored shading nets increase yields and profitability of highbush blueberries. Acta Hort., 770:193-197.

Sathisha, C.S.; Rajendra, P.; Rame-Gowda, S. and Anitalakshmi, V. (2012). Indian J. Plant Sci., 1: 35-38.

Snedecor, G.W. and Cochran, W.G. (1980). Statistical Methods. 7th Ed., Iowa State Univ. Press, Ames.

Stamps, R.H. (2008). Differential effects of colored shade nets on three cut foliage crops. Acta Hort., 770:169-176.

Stegeman, H. (1980). Retrospect on 25 years of variety identification by protein patterns and prospects for the future. Proc. Int. Seed Testing Association, 20-31.

Studier, F.W. (1973). Analysis of bacteriophage T7 early RNAs and proteins of slab gels. J. Mol. Biol., 79: 237 - 248.

Vanek, T.; Nepovim, A. and Valicek, B. (2001). Determination of stevioside in Plant Material and Fruit Teas. J. Food Comp. and Anal., 14 : 383-388.

Yadav, A.K.; Singh, S.; Dhyani, D. and Ahja, P.S. (2011). A reviewon the improvement of Stevia [*Stevia rebaudiana* (Bertoni)]. Can J. Plant Sci., 91 (1): 1-27.

Zakher, A.G. and Abdrabbo, M.A.A. (2014). Reduce the harmful effect of high temperature to improve the productivity of tomato under conditions of newly reclaimed land. Egypt. J. Hort., 4: 85-97.

تأثير بعض معاملات المناخ المعدل علي النمو الخضري، المحتوي الكيميائي الحيوي والاختلاف الوراثي بناء على البروتين ومشابه الانزيم لنبات الإستيفيا

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يعتبر نبات الإستيفيا من النباتات الاقتصادية الهامة كنبات طبي لاستخدامه كمادة محلية لمرضي السكر وفي علاج مرضي السمنة. يعتبر المناخ المعدل مؤثر هام علي انتاج المواد الفعالة في نبات الاستيفيا ويهدف هذا البحث لدراسة تاثير العوامل البيئية على الأصناف من الاستيفيا (اسبنتي – شناي وايجي١) تحت معاملات تغطية مختلفة (الشبك الأسود- الشبك الابيض والتغطية بالبلاستيك بالمقارنة بالزراعة المكشوفة)، وأشارت النتائج إلى أن استخدام المناخ المعدل عن طريق الاغطية المختلفة كان معنويا لكل القياسات مثل درجة حرارة الهواء والتربة المنزرعة و شدة الاضاءة وكذلك الرطوبة النسبية، وعلى نفس الاتجاه كانت صفات النمو الخضري والتحليل الكيميائي الحيوي لمركبي الاستيفيو سيد والريبوديوسيد كانت معنوية لجميع معاملات المناخ المعدلة. وأوضح التفريد الكهربي للبروتينات الكلية ومشابهات الانزيم (البيروكسيديز) وجود علاقات وراثية تحت تأثير معاملات المناخ المعدل والاصناف حيث اظهرت وجود ١١ و ١٣ حزمة بروتينية مختلفة بين المعاملات والاصناف علي التوالي. وكانت نسبة الاختلافات بين معاملات المناخ والاصناف وصلت لنسبة ١٠٠% حيث ظهر ١٢ و ١٣ باستخدام تحليل مشابهات الإنزيم تحت المعاملات المختلفة علي التوالي، ومع ذلك فان المعاملة بالتغطية بالشبك الأسود مع الصنف اسبنتي أوضح اعلي نسبة اختلاف وصلت لنسبة ١٠٠% باستخدام تحليل القرابة الوراثية للحزم البروتينية لمعاملات المناخ المعدل والأصناف باستخدام حسابات معدل التشابه والمسافة الوراثية باستخدام برنامج UPGMA.

الكلمات الاسترشادية: الاستيفيا، الاستيفوسيد، القطع المختلفة، التراكيب الوراثية، البروكسيديز، المشابه الإنزيمي، تحليل القرابة.