J. Plant Prot. and Path., Mansoura Univ., Vol. 4 (8): 755 - 765, 2013

RELATION BETWEEN LEAF AGE AND RESISTANCE TO CERCOSPORA LEAF SPOT DISEASE IN SUGAR BEET.

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ABSTRACT

This investigation was conducted during 2011/2012 season at Sakha Agricultural Research Station farm and screen-house belongs to Sugar Crops Institute. This investigation was carried out on Kawamera sugar beet cultivar to answer the question of why young Leaves of sugar beet possess a high level of resistance to Cercospora leaf spot? Under artificial inoculation by *Cercospora beticola* spores (50×10^3 spores / ml), disease severity, chlorophyll content, losses in both yield and sugar were evaluated in randomized complete block design experiment. Different histological and biochemical factors were measured i.e., size of stomatal apparatus, mineral contents in different leaves starting from heart leaves up to outer leaves of the plant.

Results obtained revealed that percent losses of inoculated leaves was 64.15 % in chlorophyll content, 52.05% and 41.74% in root and sugar yields were (respectively. Dimensions of stomatal apertures, diameter and stomata intensity showed that the young leaves were narrow and it has less number of stomata ranged from in inner leaves 115 stomata / mm^2 up to 331 stomata / mm^2 in outer leaves. Relative water content (RWC) was determined for sugar beet leaves, data obtained showed that young inner leaves contain less relative water content than mature outer leaves. Moreover, peroxidase activity was higher in the younger leaves than the mature one. Element analysis showed that N, P, Fe, Mn and Cu contents increased gradually from the inner first leaf and reduced in the outer fifth leaf, while the opposite was in potassium and zinc contents. Total phenol compounds were increased after inoculation; while the free and conjugated phenol compounds were higher in the young leaves than the oldest ones. This study clarified the role of leaf age and position in relation to resistance to Cercospora leaf spot disease.

INTRODUCTION

Sugar beet (*Beta vulgaris,* L) is known to be infected by many foliage diseases. The most important and destructive foliage disease is leaf spot caused by *Cercospora beticola Sacc.,* it has a wide host range which infect Spinach and some weed species (Francis, 2000). Symptoms on sugar beet were initially appear on the leaves and consist of circular spots approximately 3-5 mm in diameter showed necrotic, tea-colored with grayish centers. The lesions are dark reddish with brown border.

In presence of high relative humidity, the leaf spots has a grey, velvety appearance as spore production occurs, but in some cases, the centers of the lesions drop out, and led to shot hole symptom. Eventually, the

infected leaves collapse, but remain attached to the crown of the beet plant by the leaf stalks, possibly allowing the entry of opportunistic secondary infections into the plant. The young heart leaves are seldom to be infected by *C. beticola* (Francis, 2000) while the adult leaves are subjected to be infected by disease. The disease is of economic importance in Egypt (EL-Fahhar, 1997, EL- Sayed 2000 and EL-Fahhar, and Abou EL-Magd 2009) as well as in sugar beet growing countries (Bittner, 1999) *C. beticola* causing serious damage to the plant foliage and at least causing the total yield losses. Pool and Mckay (1916) recorded the entry of *C. beticola* in sugar beet leaves to take place through open stomata during the day time; the fungus is to be capable of penetrating the leaves through closed stomata at night. Rathaiah, (1975) found that *C. beticola* formed appressorium over the stomata when entry was made through closed stomata at night.

Varietal resistance is the best way to prevent the crop damage by *C. beticola* disease. Different anatomical features are relating to disease incidence i.e. stomata density, stomata size (length and width), duration and time of stomata opening, leaf water content, reaction between level of phenolic compounds (3- hydroxy- tyramine) in sugar beet and incidence of resistance to Cercospora leaf spot disease (Maag *et al*, 1967) Mineral leaf contents play important role in resistance and susceptibility of sugar beet plants on which Sodium, Potassium, Phosphorus, Iron, Zinc and Copper contents of the leaves establish a relationship between the level of specific minerals and resistance (Wojeiechowska and Mikolajska, 1988).

Many reports pointed out to the differences in the disease severity for the inner and outer leaves mainly for susceptible cultivar due to certain characteristics either histological and / or biochemical according to the position of the leaf from inner up to outer leaf rounds as observed by Wingard (1953), Tomiyama, (1963), Maag *et al*, (1967), Milford and Watson, (1971), Rathaiah, (1975), Mukhopahyay and Rao, (1978), Burenin and pilipenko, (1987), Mahmoud, (1992), Lamey, (1997) and EL-Fahhar, (2003). Relative water content varied according to leaf position and order. Moreover, peroxidase enzyme activity is varied in relation to leaf age as reported by Rautela, and Merle, (1970), who proved that the response of sugar beet to infection with *C. beticola* was characterized by an immediate increase in the amounts of peroxidase and ortho-diphenol oxidase and this increase was consistently higher in resistant than in susceptible varieties. So, this investigation was carried out to:

1) Determine the relation between age and position of leaf in sugar beet plant and its relation to resistance to cercospora leaf spot.
2) Relation of chemical composition of leaf mineral contents and resistance to disease.
3) Histological observations by SEM to leaf stomata in healthy leaves of inner and outer leaves of sugar beet

MATERIALS AND METHODS

This experiment was conducted at Sakha Agricultural Research Station farm during 2011/2012 growing season and in the screen house belongs to Sugar Crops Institute. Experiment with Randomized Complete Block design was followed and Kawamera sugar beet cultivar was planted in three replicated plots; each plot contain 10 rows, each row had 8 plants whereas, (50 cm) distance between rows and (25 cm) between plants within row. All recommended cultural practices were performed.

Artificial inoculation:

Samples of sugar beet plants showing cercospora leaf spot disease symptoms were collected from infected plants of early sown sugar beet fields on 2011/2012 at Sidi-Salem district, Kafr EL-Sheikh Governorate.

The sugar beet samples showing symptoms of cercospora leaf spot disease were washed carefully with running tap water, cut into small pieces, surface sterilized by immersing in 0.5% Sodium Hypochlorite (NaClO) solution for 3 minutes. The samples were washed several times in sterilized distilled water and dried between two sterilized filter papers; then transferred onto Petri dishes containing sugar beet leaves extract dextrose medium (SBLEDA) according to EL-Fahhar,(2003). For this medium, the fresh sugar beet leaf blades were sliced and 200 g was boiled in one liter of distilled water for 15 minutes and strained through double layered cheese cloth. This medium contained, beet leaf extract (100ml), Dextrose (20g) and Agar (15g). Streptomycin antibiotic was added to the media (40 ppm/L) to avoid contamination with bacteria. Plates were inoculated at 27 ± 2C˚ for 3-5 days and examined daily for the occurrence of fungal growth. The growing fungi were examined microscopically and purified using single spore technique described by Dhingra and Sinclair (1995). Pure cultures were maintained on PDA slants on 4 C˚ at refrigerator for next the inoculums, preparations for artificial inoculation. Old plants (90 days) were sprayed with adjusted inoculums 50×10^3 (conidia spores / ml) of the isolate using an atomizer. Two drops per liter of Tween 20 were added into the inoculums. Before inoculation, plants were sprayed with water to make a thin film of water on the leaf surface.

One plot sprayed with the recommended dose of Topsin M 70 (1gm / liter) was left as control. Percentage of Disease Severity (D S %) was recorded according to Shane and Teng (1992). Disease severity was recorded seven times after lesion appearance, and each score was taken every 15 days.

Root fresh weight was weighed for the whole plot in each replicate, sucrose percentage was estimated according to Carruthers, and Oldfield (1960). Total soluble solids (TSS) were determined in fresh roots of the cultivar using hand refractormeters (McGinnis, 1982), Loss percent of sucrose and root yield was determined by using the simple equation adopted by (Calpouzos, *et al* 1976) as follows:

$$\text{Loss \%} = \frac{\text{Protected Plants - Infected Plants}}{\text{Protected Plants}} \text{ X } 100$$

Total chlorophyll content of leaves was determined in mg by using chlorophyllmeter (SPAD-502) (Yoshida *et al,* 1976).

Relative water content (RWC) was determined for sugar beet leaves using the method cited by Lamattina *et al*, (2001) and calculated according to the formula:

$$RWC\ \% = \frac{\text{Sample fresh weight} - \text{Sample dry weight}^{*}}{\text{Sample turgid weight}^{**} - \text{Sample dry weight}} \times 100$$

* Dry weight was obtained after drying the samples at 80 C° for at least 48 h.
** Turgid weight was determined by subjecting Leaves to rehydration for 2 h.

Peroxidase enzyme activity was estimated according to method of Srivastava, (1987).

Histological studies was done to get data on stomatal apertures, size (length, width) on the upper epidermis of leaves and density were recorded on sugar beet cultivar of Kawamera Measurements were recorded for the fifth leaves starting from the heart leaves (the first inner leaves) up to mature leaves (Outer leaves).

Preparing specimens for Scan Electron Microscope (SEM). Scanning Electron Microscope (SEM) photographs were carried out for each sample. Using SEM Model Philips XL30 attached with EDX unit, with accelerating voltage 30 K.V, magnification 10 X up to 400000X and resolution for W (3.5 mm). Fresh leaves were taken from healthy plants before artificial infection in a screen house, at 90 days from sowing. Samples were taken from different leaves starting from heart up to the outer leaves. The samples were fixed by immersion in 4% Glutraraldehyde ($CH_2(CH_2CHO)_2$) in 0.1M Sodium Cacodylate buffer {$(CH_3)_2AsO_2Na.3H_2O$}, pH 7.3 for 4-6 h at 4 C°, post fixed in 1% Osmium tetra oxide (Os O_4) for 1 h. This was followed by washing in three changes of the same buffer over a period of 0.5 h. after post fixation dehydration of the specimens was carried out through a graded series of ethanol, 50% (10min), 70% (10 min), 80% (10 min), 95% (10min) and absolute(10min) and dried in Balzers union Critical point dryer using liquid CO_2 mounted in aluminum stubs and sputter coated with gold in a S 15 OA sputter coater and examined in a SEM Scanning Electron Microscope. (Karnovsky, 1965 and Anderson, 1966)

Determination of macro and micro elements:

Nitrogen content was determined using micro Kjeldahl method (Jackson, 1967).

Phosphorus was measured according to Johansson and Ulrich (1959) and Olsen and Dean (1965). Potassium and Magnesium measured according to Cottenie *et al* (1982).Trace elements (Zn, Fe, Cu and Mn): were measured by using atomic absorption spectrometer. This method was adopted by Jackson (1967).

Phenolic components were determined just before inoculation and 10 days after inoculation when the plants at 100 days from sowing to test the changes in phenolic components in different leaves (heart or outer).

Total phenols were determined in the ethanol extract of non infected and infected sugar beet leaves using the method described by Snell and Snell (1953).

Free phenols were determined in the ethanol extract of non-inoculated and inoculated sugar beet leaves after 10 days using Folin Ciocalteu reagent described by Bray and Thrope (1954). Conjugated phenols calculated according to the formula:

Conjugated phenols = Total phenols – Free phenols.

All obtained data were subjected to Statistical analysis was done according to Gomez and Gomez (1983).

RESULTS AND DISCUSSION

Data obtained in Table (1) showed the differences reached to 91 % in disease severity between healthy and infected plots compared with the two treatments for Kawamera sugar beet CV which show high level of susceptibility according to disease severity scale of Shane and Teng (1992).

Infected leaves by *C. beticola* showed a high reduction in chlorophyll content (64.15%) of leaves as evidence that photosynthetic activities is affected by the disease and can be affect the foliage in general that stopped all metabolic activities in late stages of the disease and cause deterioration of sugar accumulation in the roots, this can be noticed in the loss percentages in the root and sugar yield to 52.05 and 41.74 %; respectively. These results are confirmed by the results obtained by Mukhopahyay and Rao, (1978) who mentioned that the losses in root weight and sucrose due to infection with Cercospora leaf spot disease reached 33.9% and 46.4%, respectively. Lamey, (1997) recorded the losses in root yield and sucrose to 30%. Kawamera CV under study as a relatively susceptible showed a high level of susceptibility and this reflected on either reduction or loss percentages roots yield or sugar (EL- Fahhar, 1997). One of the main objective of this study is to answer an important question, why the young or heart leaves showed a high level of resistance to *C. beticola* rather than the outer or adult leaves of the sugar beet plant even in the susceptible sugar beet CV.

Stomatal apertures, diameter and number of stomata are playing an important role in increasing disease incidence. Data obtained in Table 2, and Fig's 1 and 2 showed the dimension and number of stomatal apertures of healthy leaves of Kawamera CV. The length of the stomata ranged from 8.56 µM for the heart leaves to 13.43 µM for the 5^{th} or the outer leaf, while, the stomata width was 0.63µM for the heart or the first leaf and 3.52 µM for the 5^{th} leaf of the plant. Also; data in Table (2), showed recorded stomata density 115 stomata /mm^2 in the first inner or heart leaf and 331 stomata /mm^2 for the outer or the 5^{th} leaf of the diseased plant. In general, varietal resistance as well as leaf age is considered important factors for spore penetration and infection occurrence. Length, width and number of stomata in leaves of Kawamera CV. varied from leaf to another. Likewise, heart leaves of Kawamera characterized by lower number of stomata /mm^2 and narrower stomata than the other adult leaves in the same CV. Therefore, young leaves

usually are shown free from infection with such disease. These results are in agreement with EL-Fahhar, (2003).

Table (1): Evaluation of some traits in sugar beet (Kawamera CV) toward infection with *C. beticola* during 2011/2012 growing season.

Traits	Control	Infected	Reduction loss (%)
Disease severity %	2.50	27.90	91.00
Chlorophyll content (mg)	42.40	15.20	64.15
Root weight / plant (kg)	1.46	0.70	52.05
Sucrose (%)	20.60	12.00	41.74

Table (2): Dimension and number of stomatal apertures of healthy leaves of Kawamera sugar beet C.V.

Leaf number	Healthy leaves		No. of stomata of healthy leaves stomata / mm^2
	Length(µ M)	Width (µ M)	
1st (Hart)	8.56 c d	0.63 f	115 d
2nd	8.60 d	1.30 e	160 c
3rd	9.40 c	2.11 d	205 b
4th	9.90 c	2.70 c	225 b
5th	13.43 a	3.52 a	331 a

In a column; means for each character followed by same letter are not different significantly at 5 % level by DMRT statistical analysis.

On the other hand, Rathaiah, (1975), and Burenin and pilipenko, (1987), who reported that the severity of infection depends on density of stomata and their opening size which determine the chance of the germ tube penetration. Moreover, Wingard (1953) stated that stomatal apertures of sugar beet heart leaves were too small for penetration by germ tube of *C. beticola*.

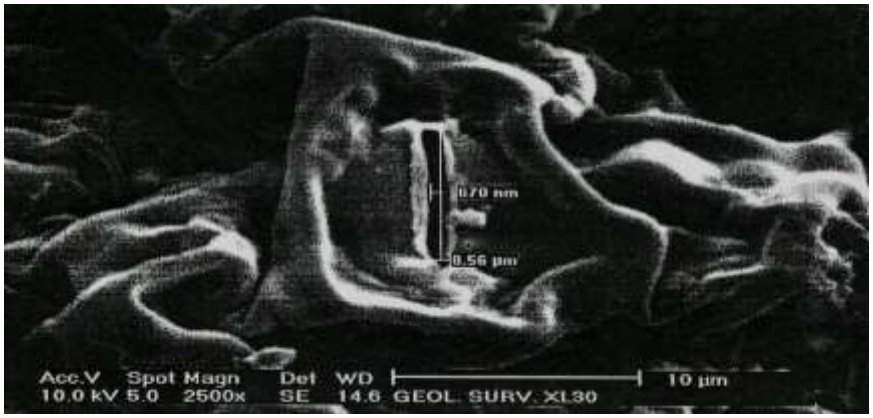

Fig (1) Dimension of stomata in healthy younger leaf of Kawamira CV.

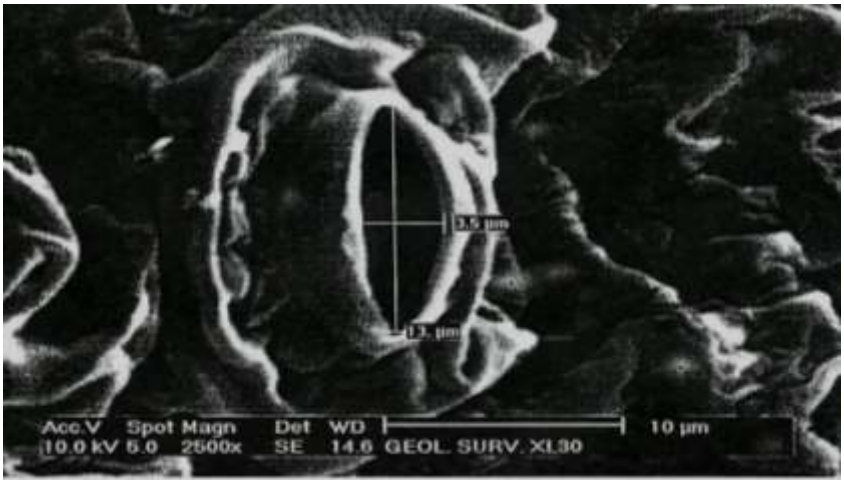

Fig (2) Dimension of Stomata size (µM) in the outer leaf healthy of Kawamira CV.

Data presented in Table (3) showed that relative water content (RWC) values of leaves was higher in the outer or adult leaves (4^{th} and 5^{th}) than the inner leaves, these results support the idea that the inner leaves acquired more resistance than the outer leaves.

Optical density values recorded for peroxidase enzyme activity after inoculation at 5 minutes intervals are varied in the first leaf (Heart) 12.35 OD to 11.75 OD for the outer leaf after 5 minutes. After 20 minutes peroxidase activity ranged from 17.85 OD for the inner leaf (Heart) to 13.88 OD for the adult or outer leaf. Obtained results explain and answer that outer leaves is less resistance than the inner or heart leaves these results are in agreement with Rautela, and Merle, (1970).

Very interesting data of elements analysis could answer the common question of why heart leaf showed resistance than the other leaves ? N, K, P, Fe, Mn, Zn and Cu contents were determined for samples of 5 leaves starting from heart leaf up to the 5^{th} or outer leaf as shown in Table (4)

Leaf nitrogen content (N %) was 0.61% in the first leaf and 3.69 % in the 5^{th} outer leaf, while potassium and Zinc contents (2.89 % and 510.1 ppm respectively) in the young leaf and (2.00 % and 255.0 ppm respectively) in the outer leaf showed an opposite trend of N., while P, Fe, Mn and Cu contents (69.10, 12.20, 68.20 and 39.50 ppm respectively) in the first leaf and (199.90, 19.20, 87.60 and 46.10 ppm respectively) in the 5^{th} outer leaf were similar in trend of N. Moreover, Zinc and Potassium contents of young leaves were higher than in adult leaves, however, these two elements particularly potassium might play a role in varietal resistance and infection occurrence. Moreover, different elements have different metabolic activities either in leaves or roots (Milford and Watson, 1971) In addition, cell size and water content of the cells increased by increasing nitrogen. Moreover, cell wall thickness turned to be thin and may enable the fungus to penetrate the leaves.

Table (3): Relative Water content (RWC), Peroxidase activity in the inner and outer leaves of sugar beet Kawamera CV

Leaf No.	RWC	Activity- OD*/min/fresh weigh (Time intervals)			
		0	5	10	20
1 (Inner)	74.75	10.70	12.35	14.24	17.85
2	76.25	10.82	12.45	14.34	17.78
3	79.00	10.90	12.75	14.54	17.88
4	84.25	10.60	11.35	12.24	13.58
5 (Outer)	92.25	10.40	11.75	12.45	13.88
LSD 0.05	3.11	0.201			

***Optical density**

Table (4): Nitrogen, Potassium, Phosphorus, Iron, Manganese, Zinc and Cupper contents in sugar beet leaves of Kawamera CV.

Leaf Ranking	Element content of sugar beet leaf						
	N (%)	K (%)	P (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)	Cu (ppm)
1st Leaf (Heart)	0.61e	2.89 a	69.10 e	12.20 e	68.20 e	510.10 a	39.50 e
2nd Leaf	1.23 d	2.45 b	110.30d	13.60 d	71.50d	355.00bc	40.10d
3rd Leaf	1.98 c	2.46 b	169.70c	15.20 c	75.20c	330.00bc	42.50c
4th Leaf	2.09 c	2.36 c	189.50 b	16.30 b	79.30b	295.00bc	44.00b
5thLeaf (outer)	3.69 a	2.00 d	199.90a	19.20 a	87.60a	255.00c	46.10a

Means followed by same letter in each column are not significantly different at 5% level by DMRT statistical analysis.

Potassium (K) plays an important role in stomatal movement either in the opening or closing as well as K increase the cell wall thickness especially the epidermal cells which increase the difficulties against the fungus to penetrate cell wall of the plant (Devllin, 1969).

Data presented in Table 5 showed that free, conjugated and total phenolic compounds were increased after leaf inoculation with *C. beticola*. Also, data showed that free and conjugated phenolic compounds were higher in the younger leaves rather than the oldest ones. Moreover, there was relation between resistance to *C. beticola* and the increase of phenolic contents in sugar beet leaves. These results are in agreement with those obtained by Maag *et al*, 1967, who reported that the phenols and other metabolites may accumulate in nearby cells of the invaded tissues and toxin as well as phytotoxins may produce phenolic glycosides that may split into more toxin glycogens. Fungal toxins may reverse their processes and host oxidizes may destroy the toxins (Tomiyama, 1963). Moreover, Mahmoud (1992) found that high concentrations of phenolic compounds have shown correlation with cercospora disease and used as measure to control leaf spot, and the resistant varieties accumulate higher total phenols than the susceptible ones.

In general, heart leaves possess narrow, thin and scattered stomata than the outer leaves. Also, young leaves contain low content of N, P, Fe, Mn, and Cu, While it have high contents of K and Zn than the old leaves.

On the other hand, free, conjugated and total phenols increased after inoculation, but the contents were high in the young than in older leaves.

Table (5): Effect of *C. beticola* on free, conjugated and total phenol contents (mg/g Fresh weight) in young and mature leaves of Kawamera sugar beet CV. before inoculation and at 10days after inoculation with the pathogen.

Leaves		Free phenols		Conjugated		Total	
		Before*	After**	Before*	After**	Before*	After**
Inner	1st leaf	48.50	57.00	93.00	96.00	140.50	152.00
	2nd leaf	46.00	53.00	92.50	95.50	137.00	147.00
Outer	4th leaf	16.70	25.20	59.00	67.10	66.30	85.80
	5th leaf	8.60	18.20	38.50	57.20	56.60	82.40

before leaf inoculation with *C. beticola
**** after leaf inoculation with *C. beticola***

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العلاقة بين عمر الورقة والمقاومة لمرض التبقع السركوسبورى فى بنجر السكر

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2- قسم النبات الزراعى – كلية الزراعة – جامعة كفرالشيخ

3- قسم بحوث الفسيولوجي والكيمياء - معهد بحوث المحاصيل السكرية – مركز البحوث الزراعية

أجرى هذا البحث بمحطة بحوث سخا الزراعية وصوبة المحاصيل السكرية فى موسم 2012/2011 وذلك بإستخدام صنف بنجر السكر كواميرا للإجابة على السؤال لماذا تُظهر الأوراق الحديثة مقاومة لمرض التبقع السركوسبورى عن الأوراق الناضجة والكبيرة ؟ وأجريت العدوى الصناعية بمعدل 50 × 10 ^{3} جرثومة / مل وأجريت التجربة باستخدام تصميم القطاعات الكاملة العشوائية فى ثلاثة مكررات وتم تقدير الشدة المرضية – ومحصول الجذور والسكر وكذا الصفات التشريحية كحجم و طول و عرض و كثافة الثغور للأوراق الحديثة والمسنة وبعض الصفات البيوكيميائية وقياس نشاط إنزيم البيروكسيديز أوضحت النتائج أن النقص فى محتوى الكلورفيل ومحصول الجذور والسكر كانت مختلفة وأوضحت النتائج أن قطر وطول الثغر وكثافة الثغور فى الأوراق الصغيرة قياساتها أقل من قطر وطول الثغر وكثافة الثغور في الأوراق الناضجة وأظهرت النتائج أيضاً أن محتوى الماء النسبى للأوراق الصغيرة أقل من المحتوى المائى النسبى للأوراق الناضجة وأن نشاط انزيم البيروكسيديز أعلى فى الأوراق الصغيرة من الأوراق الناضجة وبتحليل العناصر أوضحت النتائج أن النيتروجين و الفوسفور والحديد والمنجنيز والنحاس فى الأوراق الصغيرة تزداد تدريجياً وتكون أكثر تركيزاً فى الأوراق الناضجة عنها فى الأوراق الحديثة عكس عنصرى البوتاسيوم والزنك وبالنسبة للفينولات الكلية والمرتبطة والحرة زادت فى الأوراق الصغيرة (الداخليه) عن الأوراق الكبيرة (الخارجية) بعد العدوى.

قام بتحكيم البحث

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