Mycopath (2012) 10(2)**:** 41-49 **Research Article**

Fungal leaf spot of maize: pathogen isolation, identification and host biochemical characterization

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Abstract

Maize (*Zea mays* L.) is one of the important food crops for human and animal in Egypt as well as in the world. Current study was conducted to isolate and identify pathogen of leaf spot of maize from the infected tissue. Infected and healthy plants were also analyzed for various biochemical changes. Examination using image analysis system revealed thin and disrupted epidermis at stoma, with large size of subsidiary cells and presence of vacuoles in infected leaves. *Drechsleara dactylidis* was isolated from the diseased maize plants. Contents of chlorophyll a and b were significantly decreased, while activity of both catalase and peroxidase were significantly increased in diseased plant. Amino acids such as phosphoserine, glycine, citrulline, cystine, leucine, tyrosine and phenylalanine were decline, whereas *ß*-alanine and *ß*-amino iso-butyric acid were increased in infected plant tissue. Salicylic acid determined through HPLC found to be accumulated 2-folds and proteins expression assessed by SDS-PAGE was enhanced in infected tissue.

Key words: *Drechsleara dactylidis*, fungal leaf spot, HPLC, maize, salicylic acid.

Introduction

Maize (*Zea mays* L.) is one of the most important cereal grains grown worldwide in a wider range of environments because of its greater adaptability (Kogbe and Adediran, 2003). It is mainly used as a food source and now has become the most important raw material for animal feed (Pimentel and Patzek, 2005). Amongst the number of problems affecting maize yield, fungal diseases are responsible for causing major loss in its production. Southern leaf blight, northern leaf blight, gray leaf spots and various types of rust are amongst the major diseases of maize (Kump *et al*., 2011; Farhan *et al*., 2012). Leaf spot disease or southern leaf blight cause by *Drechslera maydis* is destructive disease of maize in warm humid areas. The fungus overwinters in corn debris as spores or mycelium. Spores are spread by wind or splashing water to growing plants. After infection and colonization, sporulation from these primary lesions serves as the source for secondary spread and infections as long as weather conditions are favorable for disease development and living tissues are present. The disease cycle may repeat every few days under ideal conditions. However, upon successful infection in host, pathogen alters the host physiology and biochemistry by disrupting photosynthesis and activity of proteins, amino acids and relevant enzymes. In response to that host defense mechanism activate and it is very imperative to understand mechanisms employed by plants to defend themselves against pathogens that may lead to novel strategies to enhance disease resistance in crop plants (Pozo *et al.*, 2005).

Inducible plant defenses such as systemic acquired resistance (SAR) are a key component of a plant's repertoire of disease resistance mechanisms and are a promising target to manipulate for improved disease control. During SAR, plants successfully resisting a pathogen can become highly resistant to subsequent infection not only by the original pathogen, but a variety of other pathogens (Navarrea and Mayoa, 2004). Three key signal molecules, namely salicylic acid (SA), jasmonic acid and ethylene mediated expression of both specific as well as basal defense responses were detected as a result of pest infection (Stout *et al.*, 2006). Although different plant species react to infection by activation of similar defensive mechanisms, their regulation may differ in important details. The accumulation of SA has been proposed as an endogenous marker for plant resistance (Klessig and Malamy, 1994). It has been documented that enhance in level of SA results in reduced susceptibility of these plants to pathogens and increases in the antioxidant activities of plants (Shahrtash *et al*., 2011). Microorganisms associated with crop plants are also capable of producing SA. According to

Enyedi *et al*. (1992), the levels of SA increased dramatically following infection of tobacco or cucumber plants with pathogens. Increases in SA levels have been correlated to changes in gene expression. Precisely how SAR leads to resistance is not completely understood, but several of the pathogenesis-related genes (PR genes) expressed during the development of resistance are antagonistic to pathogens (Navarrea and Mayoa, 2004). Currently, PR-proteins were categorized into 17 families according to their properties and functions (Van-Loon and Van-Strien, 1999). Among these, chitinases and â-1,3-glucanases are two important hydrolytic enzymes that are abundant in many plant species after infection by different type of pathogens. PR-9 is a specific type of peroxidase that could act in cell wall reinforcement by catalyzing lignifications (Passardi *et al*., 2004) and enhance resistance against multiple pathogens. PR-15 and -16 are typical of monocots and comprise families of germ in like oxalate oxidases and oxalate oxidase-like proteins with superoxide dismutase activity (Broekaert *et al*., 2000), respectively. These proteins generate hydrogen peroxide that can be toxic to different types of attackers.

Increase in level of protein is linked with activation of functioning of amino acids. Several researchers have attempted to screen the possible input of increased levels of free amino acids on resistance to disease or diseased conditions, although some free amino acids are probably related to the observed protection against the diseases (Alvin and Starratt, 1996). In this connection, there are only limited studies (Aldesuquy and Baka, 1992; Dulermo *et al*., 2009; Pawarv and Rane, 2011; Zheng *et al*., 2011), which show the increase or decrease of some amino acids in healthy and infected plants. *Fusarium solani* infection greatly influenced the total proteins and free amino acids of turmeric roots as compared to the corresponding healthy tissues (Reddy *et al*., 2005). Levels of free amino acids and total soluble protein significantly decreased in leaves and roots of tomato plants infected with *Fusarium oxysporum*, and this reduction was increased with increasing the time of pathogen infection (El-Khallal, 2007). Increase or decrease of particular amino acid after infection by the pathogen may be due to different reasons connected with either the pathogen or with the host. For example, arginine induces disease resistance via its effects on nitric oxide biosynthesis and defensive enzyme activity (Hong *et al*., 2008; Navajothy *et al*., 2011; Zheng *et al*., 2011).

This study was undertaken to assess the symptoms of leaf spot disease, to isolate and identify associated pathogen from the infected *Zea mays* plants. Diseased plants were analyzed for change in chlorophyll, catalase, peroxidase and amino acid contents. Protein was assessed through SDS-PAGE and salicyclic acid was determined by HPLC.

Materials and Methods

Isolation and identification of the pathogen

Extensive survey of healthy and diseased maize fields was carried out during July-August, 2012 in Menoufia Governorate, Egypt for investigation of disease symptoms and isolation of the pathogen. Leaf spot maize was examined thoroughly under laboratory and field conditions. A large number of diseased samples of maize plants showing characteristic symptoms of black spots mixed with few white spots on leaves were collected from farmer's fields. The specimens were brought to the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Egypt. The diseased and healthy plant samples were analyzed using image analysis system. For isolation of fungal pathogen, small pieces (2-5 mm^2) of infected leaves were directly placed on sterile Petri dishes containing potato dextrose agar (PDA) medium supplemented with streptomycin sulfate and incubated at 30 °C. Some of the infected samples were surface-sterilized with hydrogen peroxide (50%) solution. The sterilized pieces were aseptically transferred to sterile Petri dishes containing PDA medium. A portion of mycelium developing on the nutrient medium was transferred to the agar slants for purification, identification and storage for further examination. The isolated pathogen was identified on morphological basis (Ellis and Ellis, 1985; John and Brett, 2006).

Quantitative determination of chlorophylls and antioxidant enzymes

Chlorophylls content was determined according to Vernon and Seely (1966) using the following equations:

chlorophyll a (mg)/tissue (g) = 11.63 (A 665) –2.39 (A 649)
chlorophyll b (mg)/tissue (g) = 2.11 (A 649) –5.18 (A 665)

Where A denotes the reading of the optical density
Antioxidant enzymes catalase and peroxidase of healthy and infected plant were determined according to Kar and Mishra (1976).

Amino acids analysis

Cell free extracts was prepared by grinding 5 gm of each drayed healthy and infected leaves in sterile mortar using 70% (v/v) of ethanol. The slurry was centrifuged at 600 rpm for 10 min and the supernatant was concentrated using a vacuum desicator. The concentrated cell free extract was analyzed for amino acids qualitatively and quantitatively with a full automated amino acids analyzer model Le 3000 (Eppendro-Biotro Nik, Germany) at RCMB.

Extraction and quantification of salicylic acid

Salicylic acid was extracted and quantified as described by Malamy *et al.* (1992). Infected leaves were washed several times with distilled water to remove the remnants of fungal pathogen. Then 100 g of dried healthy and infected leaves were ground in 100 mL of 90% methanol and centrifuged at 6000 rpm for 15 min. The pellet was re-extracted with 3 mL of 100% methanol and centrifuged. Methanol extracts were combined, centrifuged for 10 min and dried at 40 °C under vacuum. For each sample, the dried methanol extract was re-suspended in 5 mL of water at 80 °C, and an equal volume of 0.2 M acetate buffer (pH 4.5) was added and incubated at 37 °C overnight. After digestion, samples were acidified with HCl to pH 1 and salicylic acid was extracted and back extracted with 2 volumes of cyclopentane/ethyl acetate/isopropanol (50:50:1, v/v/v). The organic extract was dried under nitrogen, re-suspended in 50 mL of 100% methanol and analyzed by HPLC at RCMB.

Protein gel-electrophoresis

Five grams of each dried healthy and infected leaves were ground in a volume of 0.1 mL sample buffer (sodium dodecyl sulfate) cracking solution. Extracts were added in 1.5 cm eppendorf centrifuge tube according to Laemmli (1970). Homogenates were heated at 95 °C for 5 min then briefly centrifuged at 12,000 rpm to pellet cellular debris. The resulting supernatants (total protein extracts) were stored at –70 °C until analysis by Polyacrylamide Gel Electrophoresis. The extract was separated by electrophoresis on 1mm thick 12.5% acrylamide slab gels. Gels were stained with Coomassie blue at RCMB.

Results and Discussion

Disease symptoms

It was found that appearance of maize fungal spots begin in months of July and August and attacks leaves, leaf sheaths, stalks, and sometimes outer husks. The first noticeable symptom of this disease develop on leaf blades and consist of black to gray small necrotic or chlorotic spots arranged as alternate bands of diseased and healthy tissue (Fig. 1A1). The appeared spots increased in size and aggregated to form lesions of about 1-5 cm in diameter when fully developed (Fig. 1A2) as compared with the uninfected plant (Fig. 1B 1 & 2) containing green leaves and stem without any spots. It was observed that disease is prevalent in hot, humid maize areas, infect plants near flowering time and can damage the crop significantly. Spots on the mid-ribs are circular and dark brown, while lesions on the laminae continue as chlorotic spots (Fig. 1A3 and 4). Current observations are supported by Parry *et al*. (1995) and Durrishahwar *et al.* (2008), who reported that temperature and humidity during pathogenesis are critical for natural fungal infections of grains.

Examination with using image analysis system of sections showed the thick epidermis (EP) in healthy leave (Fig. 2A) as compared with thin epidermis of infected leaf (Fig. 2C). The results of current study also revealed the disruption of EP at stoma (S) of infected leaves (Fig. 2D) with large size of subsidiary cells (SC) and presence of vacuoles (VC). Wanjiru *et al.* (2002) stated that cell wall degrading enzymes are involved in the colonization of host tissues by fungal hyphae. At the same time, the mesophyle tissue (MT) in infected leaves containing compact cells (Fig. 2D) compared to healthy leaves (Fig. 2C).

Isolation and identification of the Pathogen

D. dactylidis was isolated from the infected leaves. The fungus exhibited concentric colonies, with white mycelium in center with white and fluffier towards margin. Spores were olive to light brown, most were straight and some were curved and branched. Curved cell were usually 5-6 cell long with third cell distil or swollen. Straight spore measured 50 × 75 and 20 × 30 µm. Spores showed average 6 septa. Condiophore was dark measuring 100 × 10 µm with 3-4 cross septa (Zeiders, 1980).

Estimation of chlorophyll content and enzymes activity

Chlorophyll a and b (5.21 and 1.87 mg g^{-1} fresh weight, respectively) were significantly decreased and activity of catalases and peroxidases (36.23 and 0.86 μmL^{-1}, respectively) were significantly increased in infected plants (Fig. 3) as compared with healthy plant. The changes in

chlorophyll content could be linked with inactivation of enzymes responsible for its synthesis. Whereas increase in catalase and peroxidase activity could be due to activation of plant defense mechanisms upon pathogen infections (Luhova *et al*., 2006; Swarnakumari *et al*., 2011).

Estimation of amino acid concentration

There was significant reduction in the concentration and percentage of the total amount of amino acids in diseased plants as compared to the healthy one (Table 1, Fig. 4). Phosphoserine, glycine, citrulline, cystine, leucine, tyrosine and phenylalanine were less in the concentration (38.17, 3.64, 7.32, 83.27, 39.04, 49.84 and 44.51 µg L^{-1}) in infected plants compared with their concentrations (68.76, 12.25, 8.39, 110.96, 78.92, 74.67 and 106.86 µg L^{-1} respectively) in healthy plant. On the other hand, unusual amino acids like ß- alanine and ß- amino iso-butyric acid increased in concentration as a result of infection. At the same time, 4 amino acids (phosphoethanol amine, threonine, methionine and 1-methyl-histidine) were synthesized and therefore detected in infected plant only. The reduction in amino acids concentration of infected tissues may be due to their utilization by the pathogen or due to their utilization in the synthesis of proteins during host-parasite interactions (Singh and Shukla, 1987). The increase in amino acids and or the synthesis of new amino acids may be owing to an interference of host metabolism. Absence of arginine and lysine in infected plants may indicate thier role in the increased susceptibility of the plants to the pathogens. Whereas, Zheng *et al*. (2011) stated that arginine induces disease resistance via its effects on nitric oxide biosynthesis and defensive enzyme activity.

Estimation of salicylic acid (SA)-HPLC

SA was found to accumulate in infected plants as compared with the healthy plant (42.36 and 17.13 mg g^{-1} dry weight, respectively) (Fig. 5 A and B). It seems that SA levels increase in plant tissue following pathogen infection that probably results in increase in resistance of plant against pathogen (Raskin, 1992; Ryals *et al*., 1996; Durner *et al*., 1997).

SDS-PAGE

Comparisons of proteins between healthy and infected maize identified that the expression of proteins was higher in infected plant than in healthy plant, possibly associated with disease resistance. There is similarity between two proteins with molecular weight of near range of 70.61-4.2 KDa in both healthy and infected plant respectively (Table 2, Fig. 6), which probably indicate absence of their role in defense mechanism against fungal diseases. On the other hand, the appearance of high molecular weight proteins (131 and 123.22 Kda) in infected plant may be due to disease resistance or stimulation of plant-defense responses.

Fig. 1: Typical symptoms on infected (A1, 2 & 3) and healthy (B1 & 2) maize plants.

Conclusion

Spectral analysis of diseased maize leaves revealed disruption in epidermis at stoma, with large size of subsidiary cells. *D. dactylidis* was isolated from the diseased tissue. In infected tissue, chlorophyll contents and amino acid concentrations were declined, while catalases and peroxidases activitvies, salicylic acid accumulation and proteins expression increased.

Acknowledgement

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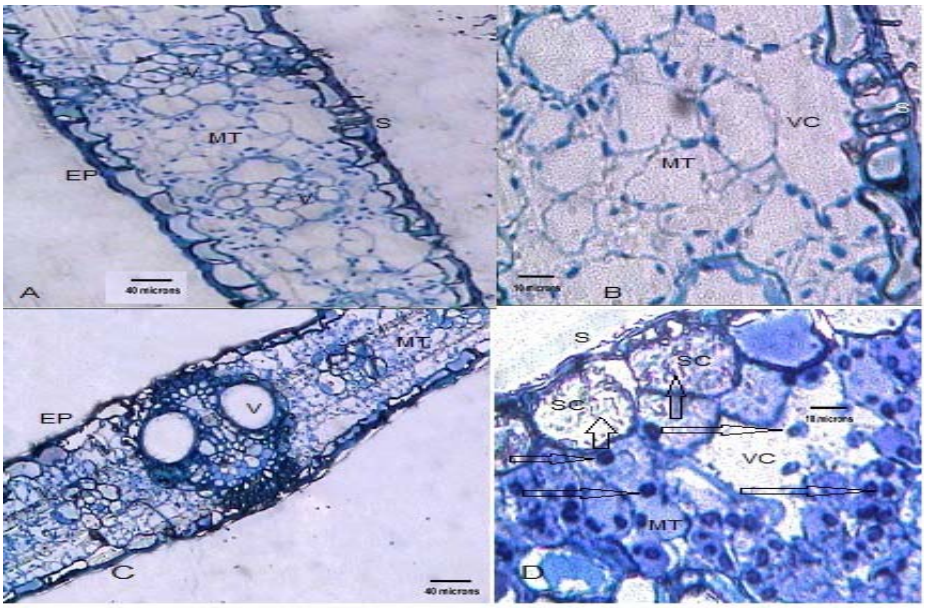

Fig. 2: Light micrographs of healthy (A & B) and infected (C & D) leaf of *Zea mays* (A & B). Leaf epidermis (EP) with stoma (S) and sub-stomatal chamber (SS); mesophyll tissue (MT) (C & D)

Table 1: Percentage and composition of free amino acids of infected and healthy maize plants.

Amino acids	Healthy plant		Infected plant	
	Concentration (µg mL^{-1})	Concentration (%)	Concentration (µg mL^{-1})	Concentration (%)
Phosphoserine	68.76	5.89	38.17	3.19
Taurine	19.05	1.63	21.34	1.78
Phosphoethanol amine	0.00	0.00	8.43	0.73
Threonine	0.00	0.00	4.97	0.42
Glutamic acid	112.8	9.60	6.91	0.58
Glycine	12.25	1.05	3.64	0.03
Citrulline	8.39	0.72	7.32	0.61
Cystine	110.96	9.51	83.27	6.95
Methionine	0.00	0.00	36.18	3.02
Leucine	78.92	6.76	39.04	3.26
Tyrosine	74.67	6.40	49.84	4.16
Phenylalanine	106.86	9.16	44.51	3.71
ß- alanine	178.65	15.31	196.55	16.40
ß - amino iso-butyric acid	212.84	18.24	315.71	26.35
1-Methyl- histidine	0.00	0.00	147.68	12.23
Carnosine	35.37	3.06	104.50	8.72
Lysine	33.14	2.84	0.00	0.00
Arginine	15.26	1.31	0.00	0.00

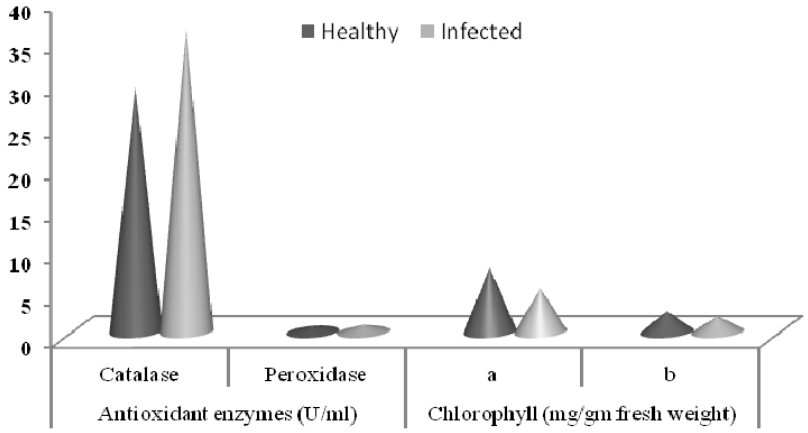

Fig. 3: Chlorophyll content and antioxidant enzymes of infected and healthy maize plants.

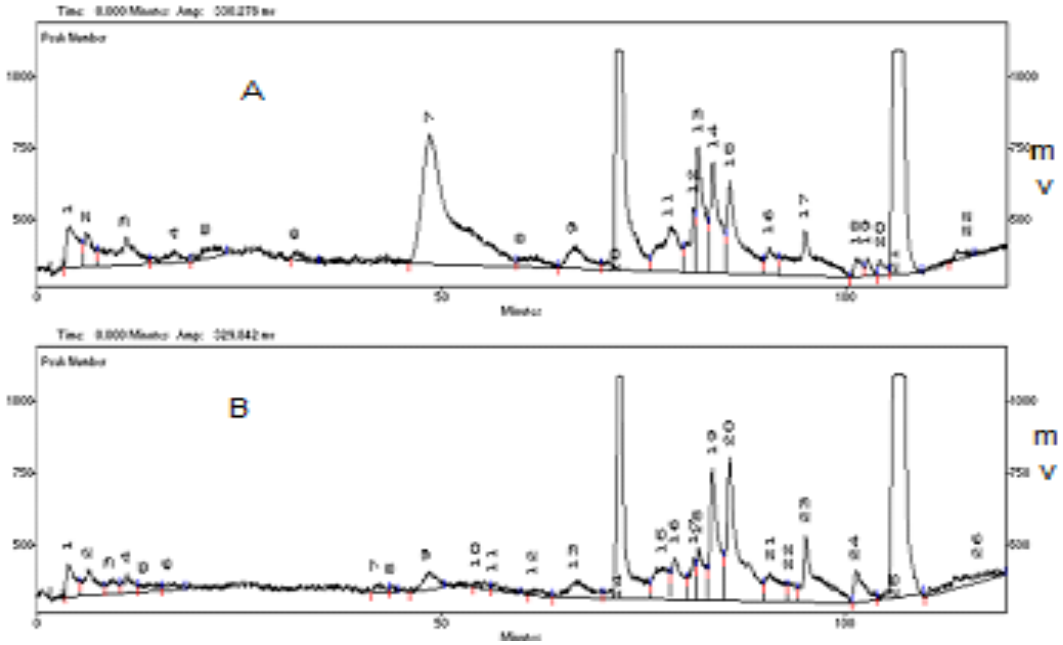

Fig. 4: Typical chromatogram of amino acid analyzer of separated amino acids in healthy (A) and infected maize plants (B).

Table 2: Molecular weight (KDa) of separated proein in infected and healthy maize plants.

Protein band code	Molecular weight (KDa) of separated proteins	
	Healthy plant	Infected plant
1	88.54	131.00
2	70.61	123.22
3	41.60	88.54
4	4.20	70.00
5	4.20	55.00
6	-	42.00
7	-	4.30

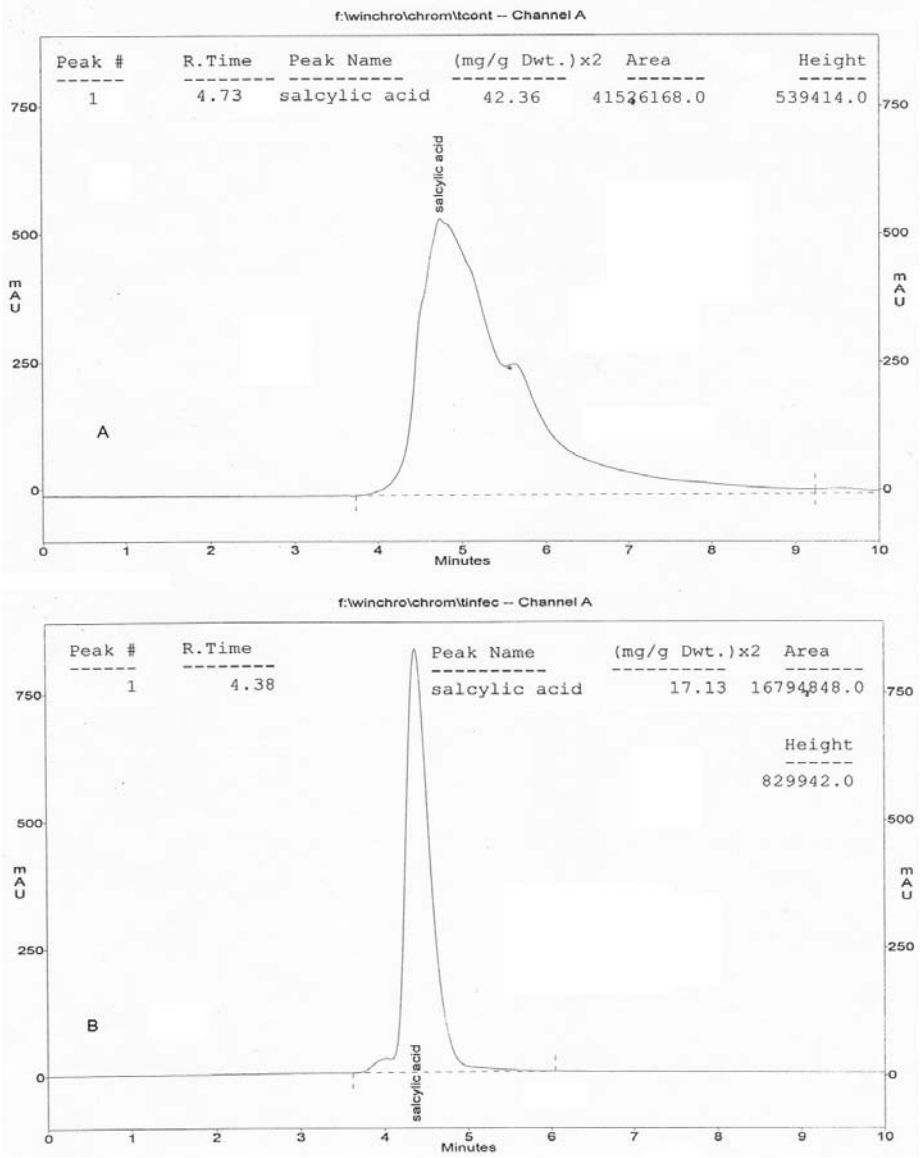

Fig. 5: HPLC chromatography showing of salicylic acid in healthy (A) and infected (B) maize plants.

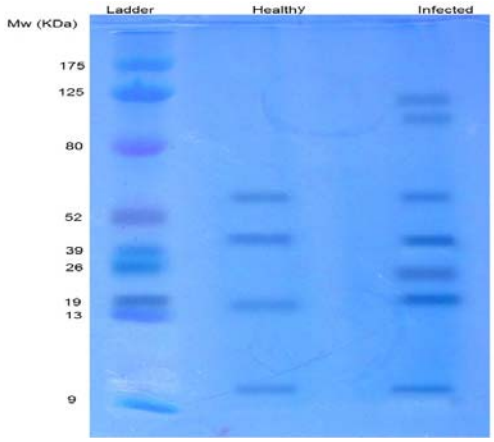

Fig. 6: Protein electrophoresis of infected and healthy *Zea mays.*

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