The Relation-Ship between Phytoalexin Production in Plant and Plant Resistance to Fungal Infection.

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Abstract

The phytoalexin accumulation in the leaves and whole plants of two cultivators of Alfalfa plants, resistant and susceptible to the fungus *Verticillium albo-atrum* isolates (V1) Virulent and (V2) AVirulent and many species were investigated, the results showed that different isolates and species of *Verticillium* fungus induce the resistant and susceptible cultivar of Alfalfa to produce different amount of phytoalexin. But the two cultivars produce similar amount of phytoalexin in response to either pathogenic isolates (V1) or the non pathogenic isolates (V2).

Introduction

Phytoalexins are low molecular weight, antimicrobial compounds, syntheses and accumulated in plants as a reaction to biotic or abiotic stress, [1][2][3] But [4] defined Phytoalexin as heterogenous group of compound that show biological activity toward activity of pathogen and considered as molecular markers of disease resistance and [5] defined Phytoalexin as any various antimicrobial chemical substances produced by plants to combat infection by pathogen (as fungus)

The possible existence of a relationship between the resistance in plant against the pathogen and phytoalexin accumulation, still a matter of controversy. Some workers consider it plays a very important role in plant resistance against the pathogen, for example, [2][6][7]while others believe that there is not sufficient evidence to indicate a clear role for example [8] Therefor this study embody the relationship between phytoalexin accumulation in the Alfalfa and the inoculation with *Verticillium* species.

The genus *Verticillium* belongs to the Class, Fungi Imperfecti (Duetromycotina), Order Moniliales (Hyphomycetes) [9]. The genus *Verticillium* comprises 40 species to date. Only five of them are considered to be pathogenic to plants, causing vascular wilt disease. They are, *V. albo-atrum*, V. *dahliae*, V. *nubilum*, V. *nigrescens* and V. tricorpus. The first two species are considered to be more economically importance than the others. [10] V. albo-atrum was first described by [11] as the causal pathogen of vascular wilt of potatoes in Germany.

Phytoalexin The concept of plants producing a defensive chemical substance has been an object of continuing interest since 1902, when [12] published his studies on disease resistance and concluded that plant cells may produce substances that allow them to overcome Pathogen attack. The result was confirmed by many workers, [7][13][14] Phytoalexin accumulation in plants was reported first in response to fungi, for example [7][15]

In 1987, [16] and co-worker, reported that over 100 phytoalexins had been identified, but [6]stated that number of phytoalexins identified has risen to over 300 so far. Most plant are able to produce a number of different phytoalexins, and any one plant family tends to produce similar phytoalexins, Phytoalexin accumulation only takeplace in response to astemulus, this astemulus as natural infection by pathogen such as fungi therefore this study involved in study the rlationship between phytoalexin production in Alfalfa plants and infection of Alfalfa by Verticillium lbo-atrum.

Materials and Methods

Isolation of the fungus. Lengths (2 cm) of stem from infected plants of Tomato and Alfalfa were surface sterilized separately by washing with 0.5 % W/V mercuric chloride for 1 minute. Segments were washed with sterile distilled water, (3 washes) and sections were sliced, using a sterile blade, directly onto plates of Dox`s agar medium or moistened filter paper held in sterile petri-dishes. Plates were incubated at 23 C^o at the dark in incubator for 3-4 days following which pure cultures of the fungus were obtained from the

resulting growth of Verticillium albo-atrum by one of two methods. Streaking` or `serial dilution. The isolates were maintained on PDA at 23 C^{o} in the dark and were renewed by subclturing monthly.

The morphology of the conidia and conidiophores was used to identify Verticillium species, according to, [9][10].

Two isolates of Verticillium albo-atrum were used. One (designated V1) was isolated from Alfalfa plants grown in the field and V2 which had been isolated from tomato, cultivar Ailsa Craig, in.

Preparation of inoculums. Spore suspensions of fungi (10^{7} spor ml-1) were prepared from 12 day old cultures grown on potato dextrose agar plates Smith et al 1995.

Method of Inoculation

A spore spreading method which described by [6] was used to inoculate leaves of cultivars of Alfalfa and root dipping method which described by [6] was used to inoculate. 5-6 weeks-old plants of Lucerne.

Extraction of phytoalexin

From leaves. A modification of the method described by [17] was used as follows; Inoculated leaves were removed from the stems, weighed then placed in a flask containing ethanol-water (2:3), (16 ml per g fresh weight). Leaves were infiltrated under vacuum by placing the flasks under vacuum for 5 minutes and repeating the process twice, after releasing the vacuum after each time. Flasks were covered with aluminum foil and placed in an orbital shaker at 100 rpm in the dark at 25 C^{o} for two hours. Suspension was filtered and the leaves were washed with distilled water (5 ml per g fresh weight). Water washings and the original filtrate were combined and the resultant solution was portioned twice against diethyl ether (0.5 ml diethyl ether per ml of extract) the resultant ether fractions were combined, and the volume reduced to 1 ml by rotary film evaporation at 30 C^{o}. The extract was transferred to a small glass vial, and the solution was evaporated to dryness under a stream of filtered air at 40 C^{o}. Samples were stored at -21 C^{o} until required.

From whole plants. Inoculated plants were uprooted from the soil and washed with distilled water to remove the remains of the soil then dried with tissue and weighed. Plants were ground with a mortar and pestle with a liquid nitrogen. The ground plant tissue was transferred to small beaker with 20 ml of 40 % V/V ethanol-water. The beaker with the mixture was placed on a stirrer for two hours, after which time the mixture was filtered through filter paper (Watman No 4). The filter paper was washed with 5 ml of 40% Ethanol V/V. The resultant filtrate and ethanol used for washing were combined with 30 ml distilled water and was portioned twice against 50 ml diethyl ether. The resultant ether fractions were combined and the volume reduced to 1 ml by rotary film evaporation at 30 C^{o}. The extract was transferred to small vial and the remaining solution was dried under a stream of filtered air at 40 C^{o}. Samples were stored at -21 C^{o} until required.

Analysis of phytoalexin

Analysis of the phytoalexin content of the samples derived from the extraction procedure was performed as follows; Samples were dissolved in 100 µl acet-nitrile (HPLC grade) containing dibutyl phthalate (DBP) as an internal standard (0.01 % V/V). After 30 minutes samples were filtered through a filter (0.45 µm ACRO Lc35 Gelman), then subjected to High Performance Liquid Chromatography (HPLC) using a Waters Millipore system composed of single Pump (model 510) and variable wave length spectrophotometer (model 455) both of which were connected to a computer system (NEC model APC IV PC).

10µl of each sample was injected via a rheodyne valve onto a column of C18 ods Silica (spherisorb ods, 5µ Hichrom, UK). The separation was performed isoatically with an eluant of acetonitrile-water (3:2), at a flow rate of 1.3 ml min-1. Compounds eluting from the column were detected by their absorption at 240 nm.

Software (Baseline, 810) was used to control pump function and to integrate the data. The retention time of medicarpin compared to the retention time of DBP, was determined by injection of an authentic sample of medicarpin that contained 0.01 % DBP in acetonitrile. This standard ratio was used to determined the identify of medicarpin in samples.

Quantitative estimation of the medicarpin.

Determining the quantity of medicarpin in the samples were achieved by using this formula [6].

$$\text{Phytoalexin} = \frac{\text{Integrated area of medicarpin peak}}{\text{Integrated area of DBP peak.}} \times \text{Response factor}$$

The resultant figure was divided by the fresh weight of the leaves (in grams), to express the amount of medicarpin as µg/g fresh weight tissue. A response factor 18.89 was determined by injection of known quantities of authentic medicarpin with 0.01% DBP and comparison the integrated area of the medicarpin with that of DBP.

Phytoalexin accumulation in the leaves of Alfalfa cultivars.

The spreading method was used to inoculate leaves of two cultivars of Alfalfa Europe (susceptible) and kabul (with a degree of resistant) with spore suspensions (10^7 spore ml-1) of one of two isolates of Verticillium albo-atrum, V1 or V2. Control leaves inoculated with sterile distilled water. Inoculated leaves were maintained at 23 C^o under a 16 hr photo-period. After 48 phytoalexins were extracted and quantified as described in Materials and Methods. One tray containing (200 leaves) was used for each treatment as follows;

Treatment I, susceptible cv.+V1.

Treatment II, susceptible cv.+V2.

Treatment III, susceptible cv. + SDW as control. Treatment IV, resistant cv.+V1 Treatment V, resistant cv. + V2, Treatment VI, resistant cv + SDW as control.

Phytoalexin accumulation in whole plants of Alfalfa in response to *V. albo-atrum* isolate V1 & V2 5-6 week old plants of two cultivars of lucerne, cv. Euver, (susceptible) and cv. Vela, (resistant) were inoculated using the root dipping method with spore suspensions (10^7 spore ml-1) of two isolates for 10 minutes. Inoculated plants were replanted in plastic pots (15 cm diameter) containing "all purpose compost" (Arthur Bowers) and maintained in the greenhouse. After 2, 5, 8, 11 and 21 days phytoalexins were extracted and quantified as described in the Materials and Methods.

Results

Phytoalexin accumulation in leaves of Alfalfa n response to *Verticillium albo-atrum* isolates V1 & V2. In order to determine whether a difference exists in the ability of V. albo-atrum, isolates V1 (pathogenic to Alfalfa & V2 (non-pathogenic to lucerne) to induce phytoalexin accumulation in leaves of lucerne cultivars, the experiment was carried The results (Fig. (1)) show that the amount of phytoalexin accumulated by cultivar Europe (susceptible) in response to isolate V2 was greater than in response to V1. Leaves of cultivar Kabul (resistant) inoculated with isolate V2 also produced a higher quantity of medicarpin than leaves inoculated with isolate V1. Leaves of the resistant and susceptible cultivars of lucerne produced similar amounts of phytoalexin in response to inoculation with isolate V1, but resistant cultivar produced higher quantity of medicarpin in response to isolate V2. The amount of phytoalexin detected in the leaves of both cultivars inoculated with sterile distilled water (control) was negligible.

Phytoalexin accumulation in whole plants of Alfalfa inoculated with V. albo-atrum isolates V1 & V2.To investigate accumulation of phytoalexin in whole plants of lucerne in response to inoculation with V. albo-atrum, isolates V1 & V2 and to examine any differences in response between cultivars, susceptible or resistant to pathogenic (V1) and non-pathogenic (V2) isolates of V. albo-atrum the following experiment was conducted.6-week old plants of cultivar, Euver (susceptible) and cultivar Vela (resistant) were inoculated with spore suspensions (10^7 spore ml-1) for 10 minutes with either of two isolate of V. albo-atrum V1 & V2. Inoculated plants were maintained in the greenhouse at 23 C^o , with a 16h photo-period. Phytoalexin was extracted and analyzed by HPLC as described in "Materials and Methods" after 2, 5, 8, 12 and 21 days following inoculation. The results are shown in Fig.(2) and (3) as follows.

Plants of cultivar Vela, accumulated similar quantities of medicarpin throughout the duration of the experiment whether they were

inoculated with V1 or V2, though after 21 days plants inoculated with V2 accumulated slightly more medicarpin.

In the case of Euver Fig.(3) the susceptible cultivar, while the amounts of medicarpin accumulated were lower than those accumulated by cultivar Vela, whether V1 or V2 were used for inoculation there was no significant difference in the amount of medicarpin accumulated.

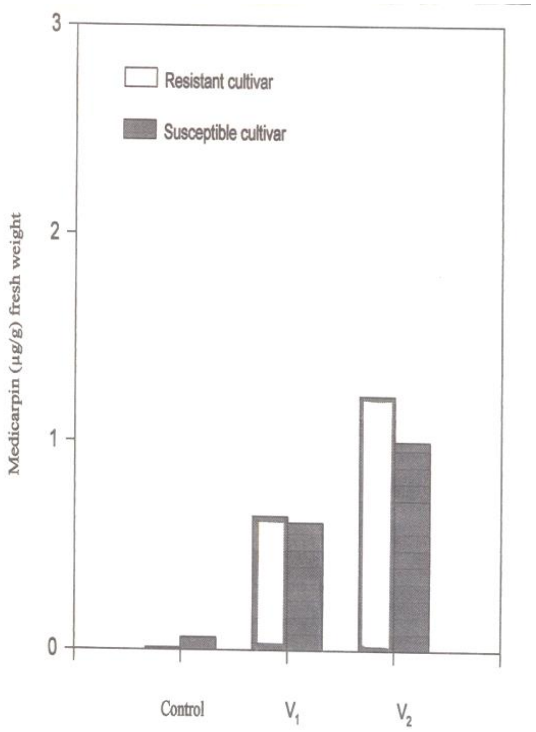

Fig.(1) Medicarpin accumulation in leaves of Alfalfa plants inoculated with the fungus Verticillium albo-atrum isolates V1 or V2. Phytoalexin extracted 48 hr following inoculation.

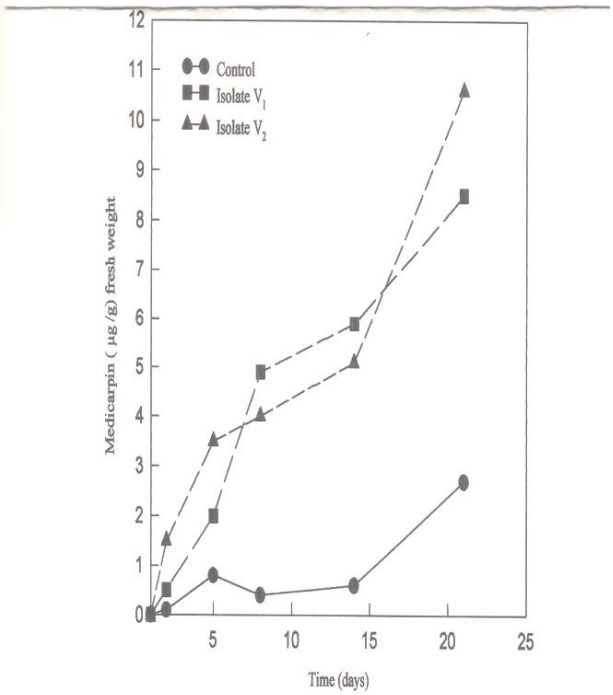

Fig. (2) Time course of medicarpin accumulation in whole plants of Alfalfa cv. Vela (resistant) inoculated with the fungus Verticillium albo- atrum isolates V1 or V2.

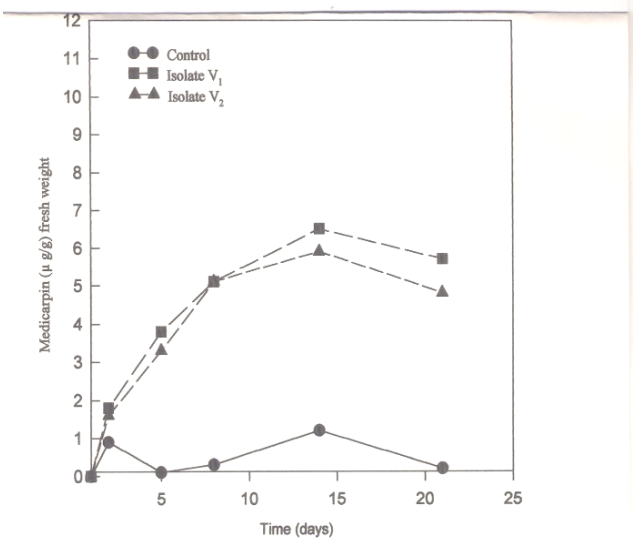

Fig. (3) Time course of medicarpin accumulation in whole plants of Alfalfa cv. Euver (Susceptible) inoculated with Verticillium albo-atrum isolates V1 or V2.

Discussion

The resistance of plants against infection by micro-organisms depends on defence systems that can be grouped in to two primary components, protection and defense. Protection refers to a static phenomenon or passive resistance. The defence component

describes a dynamic phenomenon or active defense, [6][18] Active resistance starts working when the host cells recognize an elicitor or signal from an external agent either biotic or abiotic. The plant may response in a number of ways, for example

I. Hypersensitivity response [19]

II. Deposition of callous and lignin,

III. Syntheses of phytoalexin [20][21] Phytoalexins are low molecular weight, antimicrobial compounds, accumulated and syntheses in plants as a reaction to biotic or abiotic stress, [1][22].

One of the goals of this study has been to determine whether if there is a correlation between the inoculation of Verticillium, isolates and phytoalexin production in to lucerne cultivars

Many workers have investigated formation of phytoalexins in Plants in response to inoculation with a Pathogen [2][14] variety of different methods have been used to demonstrate production of phytoalexin by members of the Leguminosae, for example, the drop diffusive technique, [23]but this method has been criticized by[17] In this study the "leaf extraction" of [24] was used to investigate production of phytoalexin in the leaves and whole plants of lucerne cultivars in response to inoculation with isolates of V. albo-atrum, pathogenic, V1, and non-pathogenic, V2. The first method was inoculation of leaves of two cultivars of lucerne, resistant and susceptible. The second method was inoculation of whole lucerne plants. The latter method is considered from the writer`s point of view, to be a natural method to investigate phytoalexin accumulation as response to inoculation with the fungi. performance liquid chromatography (HPLC) is now widely used in analyses of isoflavanols and related components, [6][25] Therefore in the present study HPLC was used to determine and analyses of phytoalexins in all experiment. In the present study the results were similar to the results of previous workers, such as, [23],[26][27].

The amount of phytoalexins produced in the leaves of resistant cultivar in response to pathogenic isolate V1 was 0.64 µg/g fresh weight whereas the figure was 1.2 µg/g fresh weight in response to non-pathogenic isolate V2. The susceptible cultivar produced only slightly lower quantities of phytoalexin in response to both, V1, 0.61 µg/g fresh weight and to V2, 1.00 µg/g fresh weight. A negligible amount of phytoalexin was produced in the control plants. However there were, clear differences in response to inoculation with V1 or V2 when whole plants were used instead of leaves. The results agree with those of many workers have studied phytoalexin production in lucerne cultivars in response to inoculation with V. albo-atrum isolates. They found that the non-pathogenic isolate induced lucerne to produce more phytoalexin than the pathogenic isolate, [23],[26][27] There are a number of possible explanations that could account for these results, such as, Either the pathogenic isolate was able to degrade the phytoalexin at greater rate than the non-pathogenic, or the non-pathogenic induced phytoalexin synthesis earlier and a greater rate than the pathogenic do. [6] stated that the differential in the elicitors from V. albo-atrum, isolate V1 and isolate V2. leads to differential phytoalexin synthesis. Because the host response to inoculation with micro-organism may depend on the presence in the elicitor preparation of several components from micro-organism, and if the micro-organisms elicitor preparation used different (lacks one or more of the components) that is affect phytoalexin synthesis. In 1995, [20] suggested that the rate of production/accumulation of phytoalexin in plant depending either on host genotypes or host and pathogen genotypes.

The following conclusions have been drawn from the results of phytoalexin experiments

i. The non-pathogenic isolate of V. albo-atrum, (V2), was induced the lucerne plant to produce phytoalexin more than the pathogenic isolate (V1) did.

ii. There is not clear connection between phytoalexin accumulation in lucerne plants and pathogenicity of Verticillium, isolates.

With regard to future research, it would be interesting to concentrate on elicitor to discover a new fungal products that act to stimuli natural plant disease resistance mechanisms to control Verticillim wilt of lucerne.

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الخلاصة

اشتملت الدراسة على استخلاص مادة الفايتوالكسين phytoalexin المتجمع نتيجة الاصابه بعزلتين كلا على انفراد من الفطر *Vertillium albo-atrum* الذى رمز له بالرمز (V1) و (V2) في ضربين من نبات الجت احدهم مقاوم للاصابة بالعزله (V1) والاخر حساس للاصابة بالعزلة (V1) وكلا الضربين مقاوم للعزلة (V2) من الفطر *V. albo-atrum* اظهرت النتائج ان العزلتين للفطر V2, V1 تحفز النبات المقاوم والحساس الى انتاج كميات مختلفة من الفايتوالكسين ولكن كلا الضربين من النبات الحساس والمقاوم ينتج كميات متساوية تقريباً كرد فعل للتلقيح بأي من العزلتين للفطر V2, V1.