

BIOCHEMICAL CHARACTERIZATION OF RESISTANCE AGAINST *Alternaria helianthi* IN CULTIVATED AND WILD SUNFLOWERS

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SUMMARY

The biochemical basis of resistance to the leaf spot/blight pathogen *A. helianthi* was compared in six wild *Helianthus* species, possessing three ploidy levels (diploid, tetraploid and hexaploid) and different degrees of resistance to *A. helianthi*, and cultivated sunflower (*H. annuus* cv. CO-4, susceptible check) in terms of sugar, phenols and isozymes of peroxidase, polyphenol oxidase and chitinase. The resistant species of wild sunflowers (*H. tuberosus*, *H. occidentalis*) possessed higher levels of constitutive as well as induced total phenols and total sugars as compare with cultivated sunflower and susceptible wild sunflowers. Polyacrylamide gel electrophoretic (PAGE) isozyme analysis of defence-related enzymes showed a positive correlation of resistance with chitinase and polyphenol oxidase and a negative correlation with peroxidase.

Key words: *Alternaria helianthi*, defence-related enzymes, *Helianthus* species, phenols, sugars

INTRODUCTION

Leaf spot incited by *Alternaria helianthi* is a major foliar disease of sunflower causing severe yield losses in the tropics and subtropics. The major limitation for genetic improvement of cultivated sunflower is the lack of acceptable levels of resistance to this disease in the cultivar germplasm. Wild sunflowers serve as potential sources for several desirable characteristics including disease resistance (Seiler, 1992). Significant variation has been reported in wild *Helianthus* species with regard to the sensitivity to *A. helianthi* and perennial wild *Helianthus* species conferring resistance to this pathogen were identified (Morris *et al.*, 1983; Lipps and Herr, 1986; Sujatha *et al.*, 1997). Before embarking upon a large-scale resist-

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ance-breeding program using wild relatives, there is a need to discriminate between host and non-host resistance because host plant resistance is genetically controlled. The importance of biochemical studies of defence reactions in the physiology of disease resistance is widely recognized. The concomitant accumulation of cell-wall-degrading enzymes like chitinase and -1-3 glucanase, alteration in the oxidative enzyme systems like peroxidase and polyphenol oxidase on induction by necrotizing pathogens and plant acquisition of resistance to further pathogenic attack have led many investigators to accept that the PR proteins are involved in the acquired resistance phenomenon (Bowles, 1990; Tomiyama, 1963).

Keeping in view the importance of *Alternaria* leaf spot in sunflower, the present study was undertaken to investigate the biochemical basis of resistance against *A. helianthi* in cultivated sunflower and wild *Helianthus* species.

MATERIAL AND METHODS

Plant material

Six wild *Helianthus* species of three ploidy levels and the highly susceptible cultivated sunflower (*H. annuus*) cv. CO-4 were used in the study (Table 1).

Table 1: *Helianthus* species used in the study

No.	Species	Accession No.	Source	Ploidy	Habit
1	<i>H. annuus</i> L.	cv. CO-4	Project Coordinating Unit (Sunflower), Bangalore, India	2n=2x=34	Annual
2	<i>H. occidentalis</i> Riddell	OCC-52	Institute of Field and Vegetable Crops, Novi Sad, Serbia and Montenegro	2n=2x=34	Perennial
3	<i>H. maximiliani</i> Schrader	MAX 11 and MAX 1631	- do -	2n=2x=34	Perennial
4	<i>H. grosseserratus</i> Martens	GRA-10	- do -	2n=4x=68	Perennial
5	<i>H. hirsutus</i> Rafinesque	HIR-03	- do -	2n=4x=68	Perennial
6	<i>H. resinosus</i> Small	RES-09	- do -	2n=6x=102	Perennial
7	<i>H. tuberosus</i> L.	TUB-04	- do -	2n=6x=102	Perennial

Initially, all the cultivated varieties and hybrids of sunflower were tested against different isolates of *A. helianthi* and since the response was uniform for all the genotypes tested and the pathogen isolate used, the study was confined to one cultivar (cv. CO-4) and the most virulent isolate of the pathogen. The wild species were obtained from the *Helianthus* species garden maintained at the Research Farm of Directorate of Oilseeds Research (DOR), Rajendraganar, Hyderabad. The leaves at the third node of newly emerged branches were used for all the experiments. For cultivated sunflower, the leaves at third node of 25- to 30-day-old plants were used.

Pathogen isolation and inoculation

The pathogen *A. helianthi* was isolated from leaves collected from infected sunflower plants grown at DOR, Rajendraganar, Hyderabad. After conducting pathogenicity tests, a single spore isolate of *A. helianthi* was maintained on sunflower leaf extract medium (SLEM) (Sujatha *et al.*, 1997). Inoculum was prepared from 15-day-old cultures of *A. helianthi* and the concentration of the suspension was adjusted to 1×10^6 conidia ml^{-1} . The leaves were spray-inoculated with the conidial suspension and covered with polythene bags to maintain high relative humidity. Plants were kept in the greenhouse at $28 \pm 2^\circ\text{C}$ and 16/8 h light/dark photoperiod. Samples were collected 3 and 24 h after inoculation (h.a.i.) for various experiments.

Estimation of total phenols

Total phenols were extracted from uninoculated (control) and leaf tissues inoculated with 80% ethanol (3 and 24 h.a.i.) and estimated using Folin-Ciocalteu reagent (Sadasivam and Manickam, 1996). Total phenols were measured as catechol equivalent after comparing with the standard curve prepared from pure catechol and expressed as g g^{-1} fresh weight of tissue.

Estimation of total sugars

Total sugars were extracted from uninoculated (control) and leaves inoculated with 80% ethanol (3 and 24 h.a.i.) and estimated according to Dubois *et al.* (1956). Total sugars were measured as glucose equivalent after comparing with the standard curve prepared from standard glucose and expressed as mg g^{-1} fresh weight of tissue.

Isozyme assays

Polyacrylamide gel electrophoresis (PAGE) was carried out for detection of peroxidase (EC 1.11.1.7), polyphenol oxidase (EC 1.14.18.1) and chitinase (EC 3.2.1.14) isozymes in control and infected (24 h.a.i.) leaf tissues of cultivated and wild *Helianthus* species. Samples of 500 mg of leaf tissue from control and inoculated material were homogenized at 4°C in 0.1 M phosphate buffer (pH 7.0) for peroxidase; 0.01 M potassium phosphate buffer (pH 7.0) with 1% Tween-80 for polyphenol oxidase and 0.1 M Tris-HCl buffer, 0.1 M MgCl_2 , 0.5 M EDTA for chitinase enzyme. The resultant homogenate was centrifuged at 10,000 rpm for 20 min. at 4°C and the supernatant was used for electrophoresis.

For detection of chitinase, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 7.5% polyacrylamide gels containing 0.01% (w/v) glycol chitin (Sigma Chem Co., USA) in resolving gel (St. Leger *et al.*, 1993). Following electrophoresis, chitinase activity was detected according to the method of Trudel and Asselin (1989). Native PAGE was carried out for detection of peroxidase and polyphenol oxidase isozymes on a 4% stacking and 7.5% resolving gel at a constant current of 35 mA per gel for 4 h. The enzymes were localized by immersing in substrate solutions as described by Sadasivam and Manickam (1996). The number of bands was counted by visual examination. Zymograms were prepared indicating the R_f values of isozymes.

RESULTS

Preliminary studies on disease severity in the six wild species and cultivated sunflower under artificial inoculation conditions (1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 conidia ml^{-1}) indicated that *H. maximiliani* and *H. grosseserratus* were susceptible and on par with cultivated sunflower. *H. resinosus* and *H. hirsutus* exhibited a moderately tolerant reaction and *H. occidentalis* and *H. tuberosus* showed resistance to the pathogen.

Estimation of total phenols

Prior to infection with *A. helianthi*, significant differences were detected in the levels of total phenols. Maximum phenol contents were recorded in *H. occidentalis* and *H. resinosus* while the lowest was recorded in *H. tuberosus* (Figure 1a). Following infection with *A. helianthi* and depending on the species, phenols content increased significantly within 3 h, continued to increase up to 24 h (*H. occidentalis*, *H. hirsutus* and *H. tuberosus*) and significantly decreased from 3 to 24 h.a.i. (CO-4, *H. maximiliani* and *H. resinosus*) and significantly decreased only 24 h.a.i. (*H. grosseserratus*).

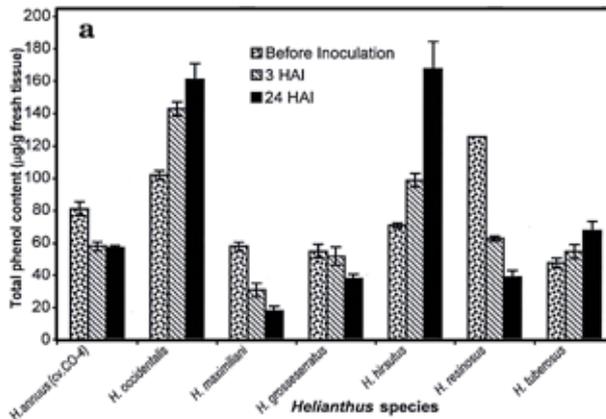


Figure 1a: Total phenol contents in cultivated and wild *Helianthus* species prior to (control) and after inoculation (3 and 24 h.a.i.) with conidia of *A. helianthi* (h.a.i. – hours after inoculation). The values are means of three replications. Bars indicate standard error of the mean.

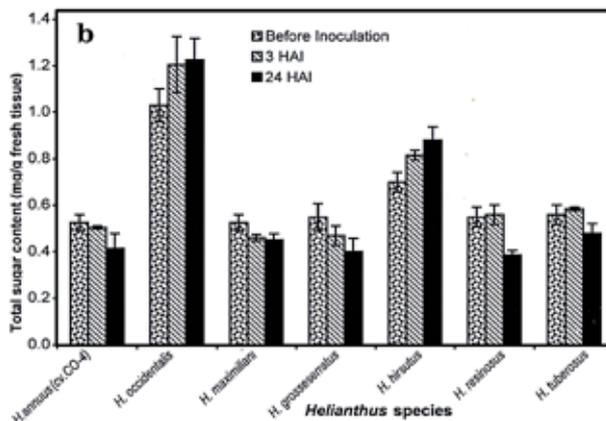


Figure 1b: Total sugar contents in cultivated and wild *Helianthus* species prior to (control) and after inoculation (3 and 24 h.a.i.) with conidia of *A. helianthi* (h.a.i. – hours after inoculation). The values are means of three replications. Bars indicate standard error of the mean.

Estimation of total sugars

The total sugar content in control (uninoculated) samples varied from 0.525 to 1.030 mg g⁻¹ fresh tissue and was greatest in *H. occidentalis* (Figure 1b). Following infection with *A. helianthi*, the total sugar content increased up to 24 h.a.i. in *H. occidentalis* and *H. hirsutus* while in the other species studied there was a constant decline or an increase 3 h.a.i. followed by a low level of induced sugars (*H. tuberosus*).

Isozyme assays

Chitinase. All the *Helianthus* species under study showed polymorphism in chitinase isozyme patterns (Figure 2).

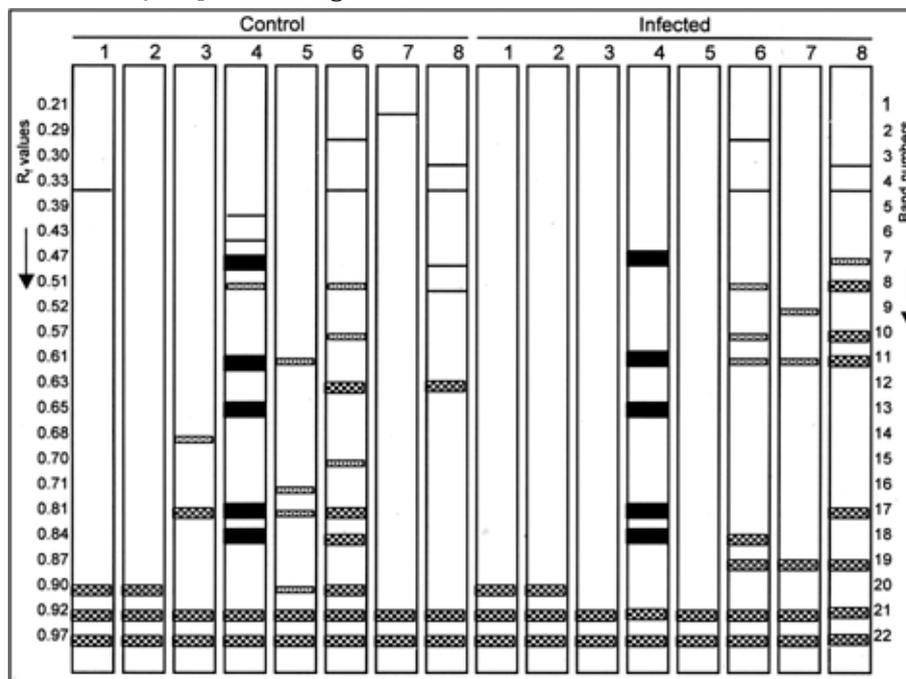
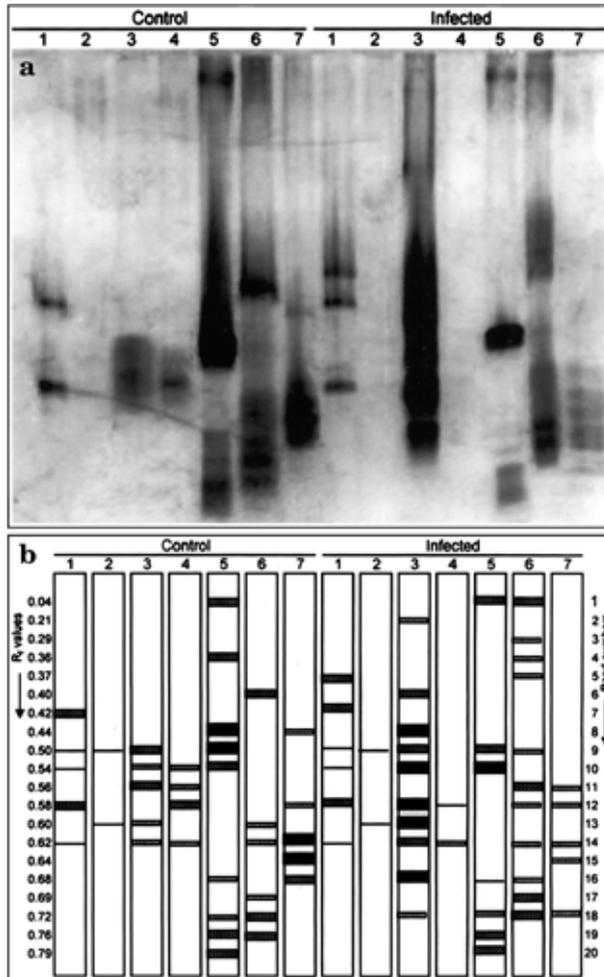


Figure 2: Zymogram of chitinase banding patterns of cultivated and wild *Helianthus* species prior to (control) and after inoculation (24 h.a.i.) with conidia of *A. helianthi*. Lanes: 1. *H. annuus* (cv. CO-4), 2. *H. occidentalis*, 3. *H. maximiliani* (Acc. Max 11), 4. *H. maximiliani* (Acc. Max 1631), 5. *H. grosseserratus*, 6. *H. hirsutus*, 7. *H. resinusus*, 8. *H. tuberosus*.

Two isozymes (R_f 0.92 and 0.97) were common to all *Helianthus* species before and after inoculation with the pathogen. Among the tetraploids, bands at R_f 0.97, 0.92 and 0.90 were found to be monomorphic in cv. CO-4. Following inoculation, absence of bands was observed in cv. CO-4 (R_f 0.33), *H. maximiliani* – Acc. Max 11 (R_f 0.68 and 0.81), *H. maximiliani* – Acc. Max 1631 (R_f 0.39, 0.43, 0.51) and in *H. grosseserratus* (R_f 0.90, 0.71, 0.61). Induction of extra bands was observed in *H.*

hirsutus (R_f 0.87 and 0.61), *H. resinosus* (R_f 0.87, 0.61, 0.57 and 0.52) and *H. tuberosus* (R_f 0.87, 0.81, 0.61 and 0.57). Absence of some bands was also observed in these three *Helianthus* species (R_f 0.81, 0.70, 0.63, 0.57; R_f 0.21 and R_f 0.63 and 0.33, respectively). No common bands were induced after inoculation by the pathogen in the studied *Helianthus* species.



Figures 3a and 3b: Gel banding patterns and zymogram of peroxidase isozymes of cultivated and wild *Helianthus* species prior to (control) and 24 h after inoculation with conidia of *A. helianthi*. Lanes:
 1. *H. annuus* (cv. CO-4),
 2. *H. occidentalis*,
 3. *H. maximiliani*,
 4. *H. grosseserratus*,
 5. *H. hirsutus*,
 6. *H. resinosus*,
 7. *H. tuberosus*.

Peroxidase. Polymorphism in peroxidase isozyme patterns was observed in all the *Helianthus* species and no monomorphic bands were observed prior to inoculation with the pathogen (Figures 3a and 3b). Among the diploid species (*H. occidentalis* and *H. maximiliani*) band at R_f 0.50 was found to be monomorphic. Both tetraploids (*H. grosseserratus* and *H. hirsutus*) showed one monomorphic band at R_f 0.54 with the cultivated sunflower cv. CO-4. Likewise, both hexaploids (*H. resinosus* and *H. tuberosus*) had bands at R_f 0.62 similar to that of cultivated sunflower.

Following inoculation, induction of one extra band (R_f 0.37) was observed in cv. CO-4, eight extra bands (R_f 0.04, 0.29, 0.36, 0.37, 0.50, 0.56, 0.58 and 0.68) in *H. resinosus* and six extra bands (R_f 0.21, 0.40, 0.44, 0.58, 0.68 and 0.72) in *H. maximiliani* indicating varied increase in the activity of peroxidase. Absence of bands at R_f 0.36 and decreased intensity of the bands (R_f 0.03, 0.50, 0.54 and 0.68) was observed in *H. hirsutus* while absence of three bands (R_f 0.21, 0.32 and 0.36) but also induction of two extra bands (R_f 0.44, 0.49) was observed in *H. tuberosus*. No bands were induced in *H. occidentalis* and no common bands were induced following infestation with *A. helianthi*.

Polyphenol oxidase. Both cultivated and wild *Helianthus* species studied showed polymorphism in polyphenol oxidase isozyme pattern (Figure 4).

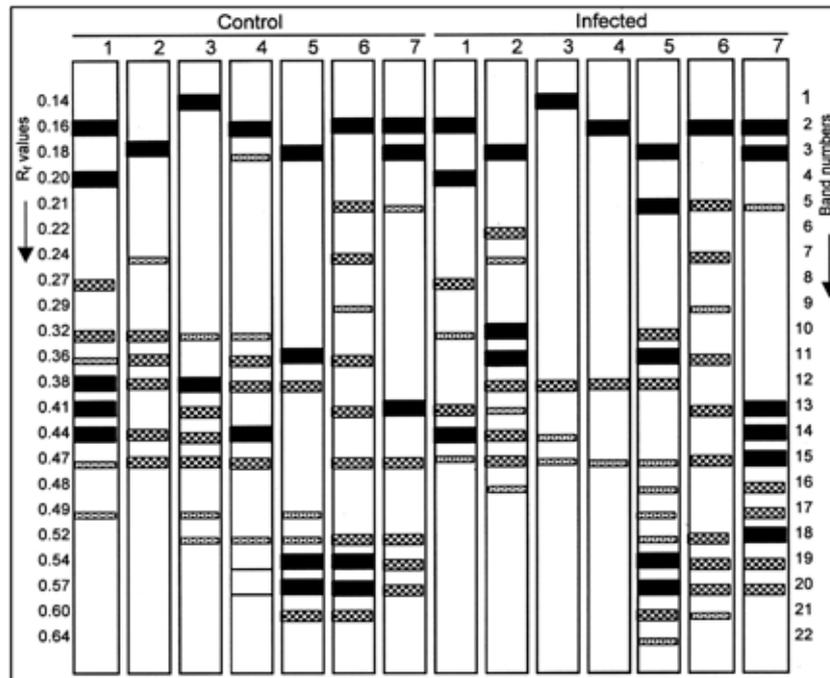


Figure 4: Zymogram of polyphenol oxidase banding patterns of cultivated and wild *Helianthus* species prior to (control) and 24 h after inoculation with conidia of *A. helianthi*. Lanes: 1. *H. annuus* (cv. CO-4), 2. *H. occidentalis*, 3. *H. maximiliani*, 4. *H. grosseserratus*, 5. *H. hirsutus*, 6. *H. resinosus*, 7. *H. tuberosus*.

Prior to inoculation, the bands at R_f 0.32, 0.38, 0.44 and 0.47 were found to be monomorphic among the diploids, the bands at R_f 0.36 and 0.38 were monomorphic among the tetraploids and the bands at R_f 0.41 and 0.47 in the hexaploids were monomorphic in cv. CO-4. Following inoculation with the pathogen, absence of bands at R_f 0.36, 0.38, 0.47 and 0.48 and decreased intensity of bands at R_f 0.32 and 0.41 were observed in cv. CO-4. The absence of bands was also observed in *H.*

maximiliani (R_f 0.38, 0.41, 0.47) while the intensity of bands was increased in *H. resinosus* at R_f 0.38, 0.41, 0.47. Induction of extra bands at R_f 0.54, 0.57 and 0.60 was observed in *H. occidentalis* (R_f 0.22, 0.41, 0.48), *H. hirsutus* (R_f 0.21, 0.32, 0.47, 0.48 and 0.64) and *H. tuberosus* (R_f 0.44, 0.48 and 0.49) while increased intensities were observed in the bands at R_f 0.47 and 0.52 in the case of *H. tuberosus*. There was no induction of common bands following inoculation.

DISCUSSION

Wild sunflowers constitute a vast reservoir of desirable characteristics including disease resistance (Seiler, 1992). Nine perennial *Helianthus* species were identified as sources of resistance to leaf blight caused by *A. helianthi* (Sujatha *et al.*, 1997). These sources belong to three ploidy groups ($2n=2x$, $4x$ and $6x$) and they possess different genomic constitutions. Hence, the mechanisms of resistance operating in these species could be expected to differ. The importance of biochemical characterization of the defence in the physiology of disease resistance is widely accepted as different mechanisms of resistance operate in different host plants (Tomiya, 1963). The present investigation sheds light on the nature of pre- and post-infectious biochemical and molecular resistance/defence mechanisms operating in six wild *Helianthus* species as compared with the highly susceptible cultivated sunflower cv. CO-4.

Variations were detected in the initial levels (before inoculation) of total phenols in leaf extracts of the *Helianthus* species under study. Very high levels were observed in some species, very low levels in others. Following infection with *A. helianthi*, the level of total phenols either increased or decreased. In cultivated sunflower and *H. grosseserratus*, the highly susceptible reaction was reflected in their lower constitutive and post-infectious total phenols. In the moderately susceptible species (*H. maximiliani* – low total phenols, *H. resinosus* – high total phenols), there was a large decline in the post-infectious total phenols. In the resistant species viz., *H. occidentalis*, *H. hirsutus* and *H. tuberosus*, regardless of the initial level, there was a high accumulation of post-infectious total phenols. Thus, in selection of genotypes for a resistance-breeding programme, it is necessary to select genotypes with higher levels of total phenols following infection with the pathogen, where phenols may play an important role as post-infectious factors in the disease resistance.

A similar trend was observed in the amount of constitutive and induced levels of total sugars. High levels of total sugars were observed in the resistant *Helianthus* species (*H. occidentalis*, *H. hirsutus* and *H. tuberosus*). An increase in total sugars was observed in species conferring a resistant reaction. The results are in agreement with those of Jayapal and Mahadevan (1969) who observed a positive correlation of the sugar and phenol levels with the degree of resistance.

Polymorphism observed in the isozyme patterns of chitinase, peroxidase and polyphenol oxidase in all *Helianthus* species could be due to the differences in ploidy and the variation in genetic constitution. The activity of chitinase and polyphenol oxidase enzymes was positively correlated with the resistance to *A. helianthi*. Decreased activity of chitinase was observed in the highly susceptible cultivated sunflower cv. CO-4, *H. maximiliani* and *H. grosseserratus*, as evidenced by the disappearance of certain bands after inoculation. Increased chitinase and polyphenol oxidase activities were observed in the resistant species *H. hirsutus*, *H. occidentalis* and *H. tuberosus*, evidenced either as the induction of new bands or an increase in the intensity of the existing bands. Similar observations were made by Tyagi *et al.* (2001) with *Alternaria triticina* in wheat. It has been reported that an endochitinase purified from barley was capable of inhibiting the growth of *Alternaria alternata* (Roberts and Selitrennikoff, 1988). An increase in polyphenol oxidase activity has been observed in infected plant tissue and was credited with blocking infection through the action of its oxidation products (Maxwell and Bateman, 1967). The *in vivo* role of the PR-proteins like chitinase and β -1,3 glucanase is to protect the host from invasion by fungal pathogen and they are integral components of a general disease resistance mechanism (Verburg and Huynh, 1997).

A negative correlation was observed between the activity of peroxidase and resistance of *Helianthus* species to *A. helianthi*. Induction of new bands was observed in the highly susceptible cultivated sunflower cv. CO-4, *H. maximiliani* and *H. resinusus* indicating enhanced activity of peroxidase in these susceptible host plants following infection with *A. helianthi*, while decreased activity was observed in the resistant sunflowers *H. occidentalis*, *H. hirsutus* and *H. tuberosus*, as the absence of pre-infection bands. In the present study, increased peroxidase activity was associated with susceptibility. Similar results were reported for maize – *Helminthosporium carbonum* interaction (Jennings *et al.*, 1969). Changes in peroxidase or rate of their synthesis may be responsible for determining resistance or susceptibility to a given plant pathogen (Fehrmann and Dimond, 1967).

The present study was aimed at discriminating the resistance sources as host and non-host types but not for a precise dissection of the host-parasite interactions at the cellular level. The results unequivocally indicate that differential defence mechanism(s) operate in response to *A. helianthi* infection in cultivated and wild sunflowers. Based on the various parameters, a high degree of resistance was conferred by *H. occidentalis* and *H. tuberosus* while a moderate level of resistance was observed in *H. hirsutus*. Perusal of the characteristics of the highly resistant species *H. occidentalis* and *H. tuberosus* indicates that the defence mechanism operating in *H. occidentalis* is a passive/incompatible reaction while that in *H. tuberosus* is an active mechanism. This is evident from the high constitutive levels of total phenols and sugars with no change in the isozymes of PR proteins in *H. occidentalis*, compared with *H. tuberosus*, where the induced levels in response to the attack of *A. helianthi* were high and bands of chitinase were observed. The success of pro-

grams of breeding for disease resistance relies on selection of appropriate material as donors. Of the two resistant species, *H. tuberosus* would be advantageous in spite of being a hexaploid, because one chromosomal set shares complete homology with the diploid cultivated sunflower. Several useful lines have been derived from this cross combination (Atlagić *et al.*, 1993).

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CARACTERIZACIÓN BIOQUÍMICA DE LA RESISTENCIA A *Alternaria helianthi* EN EL GIRASOL CULTIVADO Y SALVAJE

RESUMEN

La base bioquímica de la resistencia al patógeno de marchitamiento foliar, *A. helianthi*, fue comparada en seis especies salvajes del género *Helianthus*, en tres niveles de ploidía (diploide, tetraploide y hexaploide) que poseen diferentes grados de resistencia al patógeno estudiado, y en el girasol cultivado (*H. annuus* cv. CO-4, control sensible) en cuanto al azúcar, fenol e izoenzima de peroxidasa, polifenol de oxidasa e quitinasa. Las especies de girasol salvaje resistentes (*H. tuberosus*, *H. occidentalis*) poseían los niveles más altos de fenoles constitutivos e inducidos totales y de azúcares totales, en comparación con el girasol cultivado y especies salvajes sensibles. El análisis isoenzimático por electroforesis en un gel de poliacrilamida (PAGE) de enzimas con el mecanismo de defensa, demostró una positiva correlación de la resistencia con quitinasa y polifenol oxidasa y una correlación negativa con peroxidasa.

CARACTÉRISATION BIOCHIMIQUE DE LA RÉSISTANCE À *Alternaria helianthi* DANS LE TOURNESOL DE CULTURE ET LE TOURNESOL SAUVAGE

RÉSUMÉ

La base biochimique de la résistance au pathogène de la tache de la feuille *A. helianthi* a fait l'objet d'une comparaison à trois niveaux (diploïde, tétraploïde et hexaploïde) dans six espèces d'*Helianthus* sauvage qui possèdent différents degrés de résistance au pathogène étudié et dans le tournesol de culture (*H. annuus* cv. CO-4, contrôle sensible) pour ce qui est du sucre, des phénols et des isozymes de peroxydase, oxydase de polyphénol et chitinase. Les espèces de tournesol sauvage résistantes (*H. tuberosus*, *H. occidentalis*) présentaient de plus hauts niveaux de phénols constitutifs aussi bien que de phénols totaux induits et de sucres totaux par comparaison au tournesol de culture et aux espèces sauvages sensibles. L'analyse isozyme polyacrylamide gel électrophorétique (PAGE) des enzymes liés au mécanisme de défense a montré une corrélation positive de la résistance avec chitinase et oxydase de polyphénol et une corrélation négative avec peroxydase.

