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EFFECT OF *Sclerotinia sclerotiorum* LIB. DE BARY CULTURE FILTRATE ON SUNFLOWER MORPHOLOGICAL CHARACTERS, OXALIC ACID CONTENT AND SHIKIMATE DEHYDROGENASE ACTIVITY

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SUMMARY

Seedlings of four different experimental hybrids H1, H12, H37, H44, at the age of 28 days after sowing, were subjected to two different concentrations of culture filtrate (oxalic acid) from *Sclerotinia sclerotiorum* Lib. De Bary, while control plants were grown in the absence of toxins. Forty-eight hours after the treatment, the following characters were observed: plant height difference PHD, plant dry weight difference (PWD), basal stalk diameter difference (BSDD), oxalic acid content (OAC) in plants and shikimate dehydrogenase (SKDH) activity. Among the hybrids considered, genotype H1 appeared to be the most tolerant to *Sclerotinia sclerotiorum* culture filtrate, as confirmed by its genetic background. PWD and BSDD were unaffected by oxalic acid treatments and a significant increase in OAC and SKDH activity was observed with respect to the control.

The results of this study indicated a positive relation between oxalic acid content of fungal culture filtrate and the enzymatic activity of SKDH in sunflower plants subjected to the treatment. The oxalic acid content in plants attacked by the pathogen increases, reaching a threshold limit beyond which damage becomes visible or defense mechanisms are triggered, which implies an increase of SKDH activity, presumably related to the resistance mechanism.

The proteic structure of SKDH was also investigated and the hypothesis that SKDH alloenzyme is a monomer encoded by a single gene in a biallelic codominant locus was confirmed.

Second dimension SDS-PAGE, using a 3% ß-mercaptoethanol solution, revealed that SKDH is an isozyme characterized by two allelic forms: SkDH-a (64 kDa) and SkDH-b (57 kDa). When a 6 % ß-mercaptoethanol solution was used in correspondence of the SkDH-b band, two bands (40 and 34 kDa) were observed in the treated and control genotypes.

Key words: **Oxalic acid, resistance, *Sclerotinia sclerotiorum*, shikimate dehydrogenase, stalk rot, sunflower.**

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INTRODUCTION

Sclerotinia sclerotiorum Lib. de Bary was identified as a pathogen in *Helianthus annuus* L. by Fuckel in 1861 (Purdy, 1979) and is nowdays considered a most serious disease since it is widespread, persists for many years in the soil and has many host species (Bret *et al.*, 1993). Up to now no sunflower genotype with resistance to *Sclerotinia* has been found (Mouly and Esquerre-Tugaye, 1989), and this may also be due to the various infection modes of the pathogen, each having different genetic background on one side and the time-consuming classic breeding still in use on the other. Early techniques for screening genotypes of different plant species for resistance to *Fusarium* spp., *Verticillium albo-atrum*, *Verticillium dahlie*, "mal secco", *Phytophthora cactorum*, using their culture filtrate, have been studied (Binarova *et al.*, 1990; Buiatti and Ingram, 1991; Connell *et al.*, 1990; Nachmias *et al.*, 1990; Nadel and Spiegel-Roy, 1987, Rosati *et al.*, 1989). Several authors (Maxwell and Lumsden, 1970; Bazzalo *et al.*, 1991) observed that in sunflower and bean disease caused by *S. sclerotiorum* Lib. de Bary is associated with an accumulation of oxalic acid (culture filtrate) in infected tissue, which degrades the cell wall of the vascular tissues of stalk, thus causing the withering of the plant. Huang and Dorrel (1978) proposed the use of *S. sclerotiorum* filtrate to screen sunflower seedlings for a wilt index and Rowe (1993) measured the percent seed germination and length of germinate seedlings on clover and alfalfa genotypes exposed to exudates of *S. trifoliorum* Eriks. and *S. sclerotiorum* Lib. de Bary. These methods must however be accompanied by the fungus itself and improved by recording the oxalic acid content in the plant. Only then could they be used as an indirect estimate of the degree of infection (Noyes and Hancock, 1981).

Moreover with an amplified approach, isozymes have been the most utilized biochemical markers to study disease resistance in plants (Dry, 1985; Quillet, 1990). Shikimate dehydrogenase (E.C.1.1.1.25) is one of the most important isozymes (SKDH) studied in many species and in sunflower (Carrera and Poverene, 1995), where it seems to play a role in the resistance to *S. sclerotiorum* (Bret *et al.*, 1993). The increase in SKDH enzymatic activity is important because of its key importance in the biosynthesis of shikimic acid which is involved in the synthesis of aromatic aminoacids. These compounds are necessary to synthetize lignin for cell walls, a typical mechanical reaction of defence against fungal attacks (Buiatti, 1993; Lehninger, 1979). The utilization of isozymes markers makes it possible not only to study the defence mechanisms of the plant, but also the positive correlations observed between the expression of resistance and the increase in enzymatic activity (Mouly and Esquerre-Tugaye, 1989; Seevers *et al.*, 1971).

The paper reports the results of studies concerning : 1) the utilization of fungus filtrate of *Sclerotinia sclerotiorum* Lib. de Bary in the evaluation of sunflower variability for resistance; 2) the relation between morphological symptoms, oxalic acid

content and increase in SKDH in plants; 3) the isozyme SKDH with determination of its proteic structure and molecular weight.

MATERIAL AND METHODS

Plant material and growing method

Four experimental hybrids, obtained at Udine University, were employed and coded as follows: H1, H12, H37 and H44. The first hybrid was obtained by crossing the inbred line Max 2, coming from a population of wild species *Helianthus maximiliani*, as male parent, and the inbred line HA 61, coming from USDA-ARS, North Dakota, as female line. Both parents, as reported by ssess a high degree of tolerance to *Sclerotinia*; H12 hybrid was obtained utilizing RHA 299, restorer inbred line, (USDA-ARS, Noth Dakota), as male parent, and the inbred line HA89 (USDA-ARS, North Dakota) as female parent; hybrid H 37, by crossing inbred Max 2, as male parent, and inbred HA 89, as female parent; hybrid H 44 by crossing inbred RHA 299 as male parent and inbred HA 61 as female parent. Seed surfaces were sterilized according to the method described by Burrus *et al.* (1991). The seeds were then germinated, under controlled conditions in a growth chamber, in test tubes (130 x 25 mm) with a solid medium composed of MS salts (Murashige and Skoog, 1962), sucrose 5 g l-1 and agar 7 g l-1, at pH 5.7. Seedlings at a pair of true leaves stage were transferred and grown in hydroponic culture, utilizing the Hoagland solution, at a temperature of 20-25°C, relative moisture of about 40-50% and light intensity of about 900 $\mu E\ m^{-2}\ s^{-1}$.

Oxalic acid extraction from *S. sclerotiorum* culture

Black sclerotia of *S. sclerotiorum* coming from infected sunflower stalks and maintained on PDA at 25°C, were used. After the hyphal extension, three discs (12 mm diameter) of 14-day-old culture were transferred to Erlenmeyer flasks containing 1000 ml of the liquid medium DIFCO® and BBL® CZAPEK-DOX without agar, and then they were continuously oxygenated at a constant temperature of 27°C. After 40 days the culture solution was filtered, autoclaved at 120°C and centrifuged at 100 g for 10 min. After this the oxalic acid concentration (toxin) was colorimetrically determined using the Endpoint Sigma, 591C kit (© Sigma Chemical Co. St. Louis, MO, USA).

Experimental design and traits measured

A bifactorial completely randomized experimental design, with three replicates was used. The first factor (A) genotypes, was constituted by the four interspecific hybrids, and the second factor (B) toxin treatments, was constituted by three concentrations of oxalic acid extracted from the *S. sclerotiorum* culture. Twenty-eight days after sowing, the genotypes were inoculated with this toxin at the following concentrations: 1) 0.0 as tester, 2) 593.1 µM and 3) 1940.2 µM (Table 1).

Table 1: Formulation of each culture filtrate treatment (400 ml) and its oxalic acid concentration

Treatment	Filtrate (ml)	Liquid medium (ml)	Hoagland sol. (ml)	Oxalic acid (µM)
1	0	350	50	0
2	107	243	50	593.1
3	350	0	50	1940.2

Note: the unaltered culture filtrate derived from *S. sclerotiorum* had a concentration of oxalic acid of about 2217.4 (µM)

The experimental unit was a 400ml beaker glass with four seedlings for each genotype and treatment. The response of genotypes to toxin treatments was measured at 0 and 48 h after inoculation, for the following traits: plant height (cm), total fresh matter (g) and basal stalk diameter (cm), oxalic acid concentration OAC (µM) and shikimate dehydrogenase enzymatic activity (SKDH) (E.U. µmol sub min^{-1} mg $protein^{-1}$). To avoid the effect of the relative variability among genotypes and plants within genotypes, and to obtain absolute values, the values of morphological traits were measured before and after inoculation, plant height adjusted difference PHD (cm), plant dry weight adjusted difference PWD (g) and basal stalk diameter adjusted difference BSDD (cm), were thus obtained. The values were adjusted as follows: AV = MAI - MBI, where MAI is the measure after inoculation and MBI is the measure before inoculation. The negative adjusted values were scaled to transform them into positive ones.

Treatment method

Twenty-eight-day old seedlings were placed in 400 ml beaker glasses, with culture filtrate containing 1) 0.0, 2) 593.1 and 3) 1940.2 µM oxalic acid, obtained as shown in Table 1, and incubated at room temperature for 24h as suggested by Huang and Dorrel (1978). After the treatment, the seedlings were washed (mainly the roots) and place once more in hydroponic culture.

Extraction of leaf material

Tissues of upper green leaves were ground (0.1 g fresh matter ml^{-1} of buffer) in a mortar at 5°C. The extraction buffer solution contained: 50 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM PMFS and 2 % (v/v) ß-mercaptoethanol. After centrifugation, in Eppendorf tubes, at 5000 rpm for 5 min, the extracted supernatant was used to determine oxalic acid content, SKDH enzymatic activity and protein content.

Oxalic acid content in leaves

To determine the oxalic acid content, the Endpoint© Sigma, 591C, method was used, with 100 µl of the green leaves extract obtained as previously described.

Determination of protein content in leaf tissue

The soluble protein fraction extracted from green leaves as previously described, was also used to determine the total protein content, using the Bio-Rad protein assay kit with BSA as standard.

Shikimate dehydrogenase (SKDH) enzymatic activity assay

Shikimate dehydrogenase activity in sunflower leaves protein extract was measured using a colorimetric technique as reported by Glenner (1977) and Pearse (1972). One hundred µl of leaf extract were incubated at 37°C for 30 min in a buffer solution as described by Tanksley and Rick (1980), and brought to the final volume of 1 ml. The colorimetric reaction (tetrazolium salt) of the SKDH was stopped adding 100 µl of 25 % (w/v) trichloroacetic acid. Assay mixture in the total volume of 1 ml: 0.1 M Tris-HCl pH 8.5, 0.01 mM MTT and 2.5 % (v/v) ß-mercaptoethanol, was used as standard. The product of the reaction (tetrazolium salt) was read at 570 nm as described by Ponce *et al.* (1994).

Native PAGE

Blue native-PAGE was carried out in the Mini-Protean II cell from Bio-Rad (gel dimensions: 10 x 8 x 1.5 cm). The gels consisted of a separating gel (12 % acrylamide) and a stacking gel (4 % acrylamide) comprising 10 slots, as described by Laemmli (1970) with exception that no SDS was used. Samples of 30 µl of leaves extract, as described above, were loaded into each slot. The electrophoresis was carried out at 4°C starting at 10 mA (approximately 100 V) for 20 min and continued at 24 mA (180-500 V) for 4-5 h. The gel was than placed in a tetrazolium stain solution according to Tanksley and Rick (1980). Finally the gel was scanned as described after Sandri *et al.* (1992), using a Laser Densitometer 2222-020-Ultro Scan XL (LKB-Produkter AB).

SDS-PAGE for second dimension gel

SDS-PAGE was carried out in the protean cell from Bio-Rad (gel dimensions: 10 x 8 x 0.15 cm). Two lanes of native-PAGE (obtained before) were cut out and incubated in a denaturation solution, at two different concentrations of ß-mercaptoethanol, 3 % as described by Dabbeni-Sala *et al.* (1991), and 6 %. The lanes were briefly washed with distilled water and placed horizontally and vertically on a glass plate and fixed on a stacking gel (4% acrylamide), in a running gel (12 % acrylamide), according to O'Farrell (1975) and Laemmli (1970). The electrophoresis was carried out at 5 mA for 40 min and continued at 25 mA for 4-5 h. The gels obtained were then put in a Coomassie brilliant blue solution R250, according to O'Farrell (1975).

Statistical analysis

The obtained data were subjected to the analysis of variance, using a completely randomized bifactorial design model. The least significant difference (LSD) test was applied to those factors showing significant differences at P≤0.01 to compare means of toxin treatments and genotypes. A linear correlation matrix and a principal components analysis were used to determine the types of relations between traits.

RESULTS AND DISCUSSION

Characterization of resistance and susceptibility reactions

The analysis of variance (Table 2) shows significant differences among genotypes for traits OAC and SKDH. No significant differences were observed among genotypes for PWD, PHD and BSDD. Significant differences among inoculation treatments for all traits were observed, and the genotype-treatment interactions showed significant differences for all traits except PHD. In Table 3, genotypes H1 and H 44 had the highest values for OAC and SKDH, and H12 and H 37 the lowest.

Table 2: Mean squares of the analyzed characters obtained from adopted ANOVA model

Source	d.f	PWD	PHD	BSDD	OAC	SKDH
Genotype (A)	3	0.096	0.228	0.074	139756.2 **	0.343 **
Error a	8	0.048	0.278	0.035	11002.6	0.035
Treatment (B)	2	2.169 **	17.398 **	0.230 **	3112376.4 **	9.16 **
Interaction (AB)	6	0.142 **	0.426	0.037*	39255.9 **	0.214 *
Error b	16	0.031	0.155	0.022	6603.2	0.065
C. V *a* %		8.57	11.6	6.54	3.63	8.22
C.V *b* %		4.9	9.09	5.06	0.56	14.82

* P≤ 0.05, ** P≤0.01

The most characteristic symptoms of resistance or susceptibility to oxalic acid treatment, PWD, PHD and BSDD, revealed that the most resistant genotype was H1. This genotype had the lowest values of PWD, PHD and BSDD before the exposure to fungal toxin, but after inoculation by the second and third treatments, showed the highest values. The PWD values, in particular, and BSDD value, in correspondence of the two treatments with toxin, revealed no significant difference from the values of their controls.

Genotypes H12 and H37 developed thick stalks (BSDD) when subjected to the second treatment, but after the third treatment their resistance collapsed, as confirmed by the values of BSDD, which were significantly lower.

The screening of genotypes according to their basic morphological traits within short time from treatment (48 h) has to be supported by OAC and SKDH analysis, more suitable to detect small variations on plant mechanism developed in a short time.

Table 3: Mean values and least significant differences of morphological and biochemical traits for the factors: genotypes (A), oxalic acid treatments (B) and their interactions (AB)

Factor	PWD (g)	PHD (cm)	BSDD (cm)	OAC (µM)	SKDH†
1. H1 (A)	1.14	1.97	1.19	844.84	1.18
2. H12	1.08	2.16	1.13	712.66	0.99
3. H37	1.17	2.22	1.00	622.41	0.76
4. H44	1.33	1.89	1.20	896.17	1.17
L.S.D $p \leq 0.01$	n.s.	n.s.	n.s.	165.9	0.30
1. 0.0 (T) (B)	1.63	3.45	1.21	411.56	0.24
2. 593.1	1.12	1.33	1.21	543.36	0.88
3. 1940.2	0.79	1.40	0.97	1352.14	1.96
L.S.D $p \leq 0.01$	0.21	0.47	0.20	96.9	0.30
1.1 (AB)	1.32	2.96	1.23	443.60	0.16
1.2	1.26	1.64	1.26	705.42	1.28
1.3	0.85	1.32	1.04	1385.50	2.10
2.1	1.59	3.93	1.20	379.33	0.28
2.2	0.98	1.23	1.27	502.33	0.80
2.3	0.68	1.33	0.92	1256.33	1.92
3.1	1.54	4.00	1.00	340.38	0.21
3.2	1.17	1.25	1.14	417.29	0.66
3.3	0.79	1.42	0.81	1109.57	1.41
4.1	2.08	2.92	1.38	482.94	0.29
4.2	1.06	1.22	1.18	548.40	0.79
4.3	0.83	1.52	1.06	1657.17	2.42
L.S.D $p \leq 0.01$	0.46	n.s.	0.36	214.25	0.56

† (E.U. µmol sub. min^{-1}mg $protein^{-1}$)

The OAC and SKDH variations in the genotypes subjected to oxalic acid treatments were analysed and reported in Table 3. Among the hybrids analysed, H1 showed the greatest and most significant increase in quantity of oxalic acid in the plant with respect to the control (more that 59 %) when subjected to 593.1µM of oxalic acid treatment, whereas SKDH activity increased from 0.16 to of 1.28 E.U., significantly higher than its control.

H 44 did not reveal a significant increase in OAC (13.5 %) and SKDH activity (172,4%). Hybrids H 12 and H 37 had a 32.4 and 22.6 % increase in oxalic acid content with respect to their controls, and a 585.7 and 571.4 % increase in SKDH activity (0.80 and 0.66 of E.U., respectively).

All genotypes, when subjected to the highest concentration of oxalic acid (1940.2 µM) showed significantly higher values of OAC and SKDH activity than those of the controls and of the second treatment.

H 44 in particular had the highest quantity of oxalic acid (1657.2 µM, corresponding to about 243.1 % of its control) and H1, H 12 and H 37 had 1385.5 µM, 1256.33 µM and 1109.6 µM respectively, (212 %, 231.2% and 226% of their controls, respectively).

The SKDH enzymatic activity values were higher in H 44 (2.42 E.U.) but were not statistically different with respect to H 1 (2.1 E.U.) and H 12 (1.92 E.U.). H 37 had the lowest value of SKDH (1.41 E.U.). However, the percentage increase in SKDH activity was highest (1212.5%) in H1, greater than in H 44 (734.5 %), H 12 (585.7 %) or H 44 (571.4).

Oxalic acid is naturally present in sunflower plants, but it increases with the pathogen attack reaching a threshold limit beyond which it induces damages or can trigger the defence mechanism of the plant (Buiatti,1993; Marciano *et al.*, 1983).

The results of this work support the hypothesis by Bret *et al.* (1993), according to which the sunflower defence mechanism to *S. sclerotiorum* implies an increase in SKDH enzymatic activity and that it is related to an increase in thickness and hardness of the cell wall (Bret, 1991). In fact, as reported above, the increase in oxalic acid observed in H 12 induced only a small increase in SKDH activity and the plants showed marked symptoms on the main growth characters, as previously observed by Enferadi-Tahmasebi *et al.* (1997).

The combination of the inoculation method, using fungus inoculum, and the evaluation of responses to fungus culture filtrate based on an early evaluation in changes of morphological traits, oxalic acid content and SKDH activity, could be a starting point to set up a suitable early method to screen resistant sunflower genotypes to *Sceriotinia sclerotiorum*.

Morphological and biochemical resistance-susceptibility systems

The principal components analysis identified two types of association between morphological and biochemical traits association, for the tester and for treatments.

Table 4: Latent vectors for morphological and biochemical traits in descending order of importance at Prin1 in control plants

Trait	Prin 1	Prin 2	Prin 3
Basal stalk diameter difference	0.538	-0.055	0.547
Oxalic acid concentration	0.523	-0.269	0.018
Plant weight difference	0.409	0.481	-0.706
SKDH activity	0.264	0.690	0.372
Plant height difference	-0.447	0.467	0.254
Latent roots	3.237	1.572	0.191
Variance %	64.74	31.431	3.829
Cumulative Var. %	64.74	96.171	100

The latent vectors for the tester are shown in Table 4, where the first principal component, that explained 64.74 % of the total variation, was composed of BSDD and OAC. The second component explained 31.43 % of the total variation, and it was composed of SKDH, PWD and PHD. This component could be interpreted as growth regulation. The third component, that explained 3.83 % of the total variation, was composed by BSDD and SKDH. The latent vectors for the treatments are shown in Table 5. In this case the first four principal components explained 84.3 %

of the total variation. The first principal component explained 65.77 % of the total variation and was composed by OAC and SKDH. The second component explained 25.25 % of the total variation and was composed by PHD, PWD and BSDD. The third component explained 7.1 % of total variation, and it was composed by BSDD and SKDH. The fourth component explained 1.71 % of the total variation, and it was composed by PWD and SKDH.

Table 5: Latent vectors for morphological and biochemical traits in descending order of importance at Prin1 in plants submitted to the toxin treatments

Trait	Prin 1	Prin 2	Prin 3	Prin 4
Oxalic acid content	0.534	0.141	0.284	0.247
SKDH activity	0.511	0.237	0.417	0.271
Plant height difference	0.181	0.803	-0.431	-0.369
Basal stalk diameter difference	-0.446	0.332	0.742	-0.363
Plant weight difference	-0.472	0.411	-0.094	0.773
Latent roots	3.288	1.263	0.355	0.085
Variance %	65.77	25.251	7.104	1.708
Cumulative Var. %	65.77	91.021	98.125	99.833

Table 6: Linear correlation matrix among morphological and biochemical traits. Plants submitted to the toxins treatments above (n=24, $\overline{X}$n=8) and control plants below (n=12, $\overline{X}$n=4)

	PWD	PHD	BSDD	OAC	SKDH
PWD		-0.697	0.367	0.595	0.343
PHD	-0.273		-0.890	-0.988 **	-0.904 *
BSDD	0.597	-0.792		0.950	0.989 **
OAC	0.487	-0.953 *	0.937 *		0.958 **
SKDH	0.821	0.142	0.440	0.158	

* $p \leq 0.05$, ** $p \leq 0.01$

The genotypes cultivated as controls showed the plant OAC was significantly and negatively correlated with PHD and positively correlated with BSDD (Table 6). In this system the SKDH activity was independent from the plant OAC. If the higher OAC content in control plant could be associated to a greater degree of tolerance, the hypothesis that the susceptible genotypes are characterized by weak and tall seedlings and the resistant genotypes by stronger and shorter ones could be made.

The genotypes subjected to the treatments with the toxic filtrate culture showed that the plant OAC was significantly and positively correlated with SKDH activity (0.958), confirming the strong relationship between these two characters. The latter was significantly correlated with BSDD (0.989) and both OAC and SKDH were significantly and negatively correlated with PHD (Table 6).

Study of the SKDH alloenzyme forms

As can be observed in Figure 1, two bands were evident in all genotypes, both treated and non-treated with fungus oxalic acid extraction. This result indicates the

presence of two different alleles for the SkDH enzyme (SkDH-a and SkDH-b) in the parents of the hybrids analyzed, confirming the hypothesis of many authors (Polley, 1978; Soltis and Soltis, 1986) that SKDH is a monomer enzyme coded by a single gene in a single biallelic codominant locus.

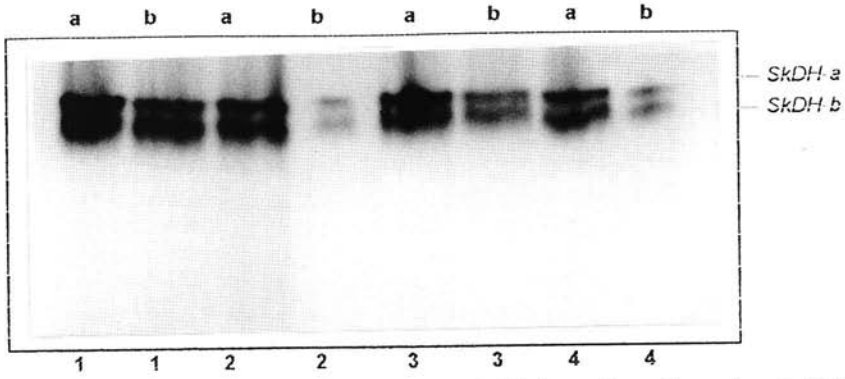

Figure 1: Alloenzyme patterns for SKDH enzyme in 28 days old sunflower leaves. SkDH-a and SkDH-b represent the two allelic forms of the isozyme.
a, plants submitted to oxalic acid (1940 µM) 48 hours after treatment and b, plants as controls. 1, 2, 3, 4 are H1 hybrid, H12 hybrid, H37 hybrid and H44 hybrid, respectively. In correspondence of the genotypes subjected to the toxin the bands are more marked than in the controls.

The bands observed in all genotypes subjected to oxalic acid extract were better marked with respect to their controls. This observation coincides with the data shown in Table 3, thus the grade of tinction is proportional to SKDH enzymatic activity.

Structure and molecular weight of SKDH

In order to rule out the possibility that the above forms were due to an artifact during electrophoresis, a second dimension SDS-PAGE was carried out, using a 3% concentration of ß-mercaptoethanol. In Figure 2 a double band is evident, confirming that the isozyme form of SKDH is coded by two different alleles, SkDH-a and SkDH-b. This result pointed out a possible bifunctinality of the enzyme, confirming the postulate published by Polley (1978). Moreover, Figure 2 shows that the allelic form SkDH-a codes a polypeptide with a molecular weight of about 64 kDa and the allelic SkDH-b codes for a polypeptide with a molecular weight of about 59 kDa.

When the concentration of ß-mercaptoethanol was increased to 6.0% (Figure 3), the band codified by allele SkDH-a remained almost unvaried with respect to that of Figure 3 (around 62 kDa of molecular weight). On the other hand, two bands with the molecular weights of around 40 kDa and 34 kDa, in correspondence to the pre-

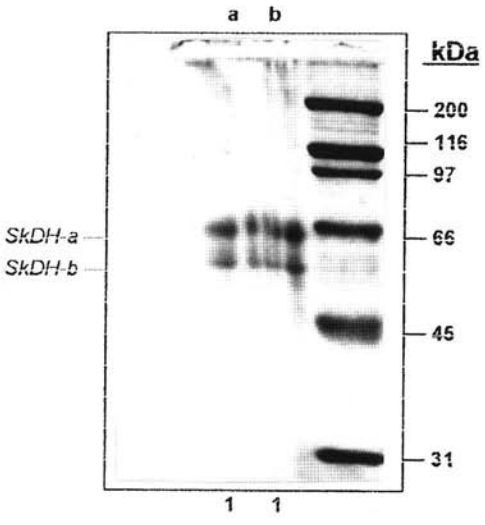

Figure 2: Second dimension SDS-PAGE for the isozyme SKDH with 3% β-mercaptoethanol. The two stained bands were cut from the gel of first dimension Native-PAGE (in Figure 1) and then incubated at 3% β-mercaptoethanol. SkDH-a and SkDH-b are the two allelic forms, showing bands with molecular weights of about 64 and 57 kDa, respectively. a, plants submitted to oxalic acid (1940 μM) 48 hours after treatment and b, plants as controls. 1 is H1 hybrid.

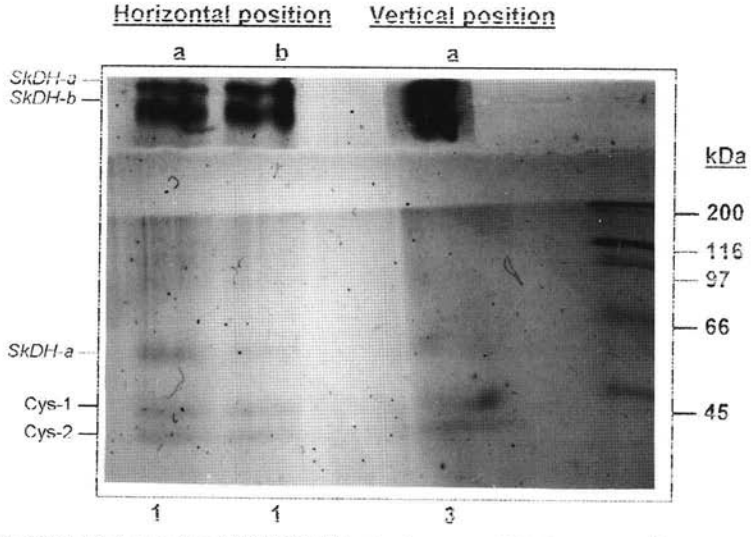

Figure 3: Second dimension SDS-PAGE for the isozyme SKDH with 6% β-mercaptoethanol. The two stained bands were cut from the gel of first dimension Native-PAGE (in Figure 1) and then incubated at 6% β-mercaptoethanol. SkDH-a shows a band with a molecular weight of about 62 kDa, while SkDH-b results composed by two residues instead of the previous single band: Cys-1 and Cys-2, with a molecular weight of about 40 and 34 kDa, respectively.
H1 hybrid fixed in horizontal position (1) and H37 hybrid fixed in vertical position (3) as reference position; plants submitted to oxalic acid (1940 μM), 48 hours after treatment (a) and plants as controls (b).

vious single band codified by SkDH-b allele, were observed in horizontal and vertical position (Figure 3).

Probably the polypeptide chain coded by SkDH-b contains one sulfhydryl -SH group, not observed in SkDH-a. This disulfide bridge perhaps was reduced by an excess ß-mercaptoethanol which converted it in two residues of cysteine. The residues of cysteine, located in the main or lateral chain, have an important role in the catalytic activity (Lehninger, 1979). The increase in catalytic activity of SKDH and its relation with oxalic acid content could probably be related to the resistance mechanism, and should therefore be studied in more detail, opening new prospectives in breeding efficiency for pathogen resistance.

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EFECTOS DEL FILTRADO DE CULTIVO *Sclerotinia sclerotiorum* LIB. DE BARY EN LOS CARÁCTERES MORFOLÓGICOS, CONTENIDO DE ÁCIDO OXÁLICO Y LA ACTIVIDAD DEHIDROGENASA DE SHIKIMATO EN EL GIRASOL

RESUMEN

Se sometieron plantones de cuatro híbridos experimentales distintos H1, H12, H37 y H44 a dos concentraciones distintas de filtrado de cultivo (ácido oxálico) de *Sclerotinia sclerotiorum* Lib. de Bary, mientras que las plantas del control fueron cultivadas en ausencia de toxinas. A las 48 horas después del tratamiento, se determinaron los siguientes carácteres: diferencias en altura de las plantas DAP, diferencias en el peso seco de las plantas (DPS), diferencias en el diámetro del tallo basal (DDTB), el contenido de ácido oxálico (COA) en las plantas y la actividad dehidrogenasa del shikimato (DHSK). Entre los híbridos considerados, el genotipo H1 parecía ser el más tolerante en un filtrado de cultivo *Sclerotinia sclerotiorum* según se confirmó mediante su origen genético (véase Materiales y Métodos). DPS y DDTB no resultaron ser afectados por los tratamientos con ácido oxálico y se observó un aumento significativo del contenido de ácido oxálico y de la actividad del DHSK con respecto a las plantas de control.

Los resultados de este estudio han hecho que se destacara una relación positiva entre el contenido de ácido oxálico y la actividad enzimática de DHSK en plantas de girasol sometidas al tratamiento. El contenido de ácido oxálico aumenta en plantas agredidas por el patógeno, alcanzando un limite umbral más allá del cual los da(os se hacen visibles, o bien se disparan los mecanísmos de defensa, 10 que implica un aumento de la actividad de DHSK, que está presumiblemente relacionada con el mecanismo de resistencia.

También se investigó la estructura protéica del DHSK y se confirmó la hipótesis de que el aloenzima DHSK era un monómero codificado por un sólo gen en un lugar codominante dialélico.

Un SDS-PAGE de segunda dimensión, utilizando una solución de 3% ß-mercaptoetanol, mostró que DHSK era un isoenzima caracterizado por dos formas alélicas: DHSK-a (64 kDa) u DHSK-b (57 kDa). Cuando se utilizó una solución de 6% ß-mercaptoetanol en correspondencia a la banda DHSK-b, se observaron dos bandas (40 y 34 kDa) en los genotipos tratados y en los del control.

EFFETS D'UN FILTRAT DE CULTURE DE *Sclerotinia sclerotiorum* LIB. DE BARY SUR LES CARACTÈRES MORPHOLOGIQUES DU TOURNESOL, LA TENEUR EN ACIDE OXALIQUE ET L'ACTIVITÉ SHIKIMATE DESHYDROGÉNASE

RÉSUMÉ

Les plantules de quatre hybrides expérimentaux H1, H12, H37 et H44 ont été soumises à deux concentrations de filtrat de culture (acide oxalique) de *Sclerotinia sclerotiorum* Lib. de Bary, tandis que les plantes témoins étaient cultivées en l'absence de toxines. Les caractères suivants sont observés 48 heures après le traitement: différence de taille (PHD), différence de poids sec (PWD), différence de diamètre de la base de la tige (BSDD), teneur en acide oxalique des plantes (OAC) et activité shikimate deshydrogénase (SKDH). Parmi les hybrides étudiés, le génotype H1 apparaît le plus tolérant au filtrat de culture de *Sclerotinia sclerotiorum*, comme le suggère son fonds génétique (voir matériels et méthodes). Les PWD et le BSDD ne sont pas affectés par les traitements acide oxalique et une augmentation significative d'OAC et de l'activité SKDH est observée commparativement au témoin.

Les résultats de cette étude soulignent une relation positive entre la teneur en acide oxalique et l'activité enzymatique de la SKDH chez les plantes de tournesol soumises au traitement. La teneur en acide oxalique des plantes attaquées par le pathogène augmente, atteignant un seuil limite au delà duquel les dommages deviennent visibles ou les mécanismes de défense se déclenchent, ce qui conduit à une augmentation de l'activité SKDH, que l'on présume liée au mécanisme de résistance.

La structure protéique de la SKDH a été également étudiée et l'hypothèse selon laquelle l'allozyme de la SKDH est un monomère codé par un simple gène à un locus diallélique codominant est confirmée.

Une analyse en deux dimensions SDS-PAGE, avec une solution d'ß-mercaptoethanol à 3%, révèle que la SKDH correspond à un isozyme caractérisé par deux formes alléliques: SKDH-a (64 kDa) et SKDH-b (67 kDa). Lorsque une solution à 6% de ß-mercaptoethanol est utilisée en relation avec la bande SKDH-b, deux bandes (409 et 34 kDa) sont détectées dans les génotypes traités et témoins.