COMPARISON BETWEEN ARTIFICIAL INOCULATION AND CULTURE FILTRATE OF *SCLEROTINIA SCLEROTIORUM* LIB. DE BARY TREATMENTS ON NINE SUNFLOWER GENOTYPES.

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Abstract
Nine sunflower genotypes (AC2221, 7Ro, C, TENOR, HA89, AC4122, R28, R 978 and Cina2) were tested for their response to artificial inoculation with mycelium and culture filtrate of *Sclerotinia sclerotiorum* (Lib.) de Bary. The reaction of the genotypes was measured as the reduction in some growth characters compared with their untreated controls and leaf area and plant dry weight were found to be the most appropriate characters for screening genotypes. Of the genotypes studied, AC 4122 and R28 were moderately tolerant, while C, HA 89 and TENOR highly susceptible. The culture filtrate induced the development of disease symptoms very similar to that of the artificial inoculation, with the exception of basal stem diameter where an enhanced growth and intense lignification by the pathogen infection was observed. Both treatments determined a very large increase in the levels of oxalic acid in the leaves and this increment, expressed as a percentage with respect to the control plants, can be used to screen for tolerance to *Sclerotinia,* using the culture filtrate. In contrast, even though an increased activity of shikimate dehydrogenase (SKDH) in the treated plants was observed for both treatments, this enzyme appeared to be triggered by a more specific reaction between mycelium and host relationship and not simply induced by the increase of oxalic acid in the tissue.

Introduction

In the temperate regions of the world, white rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is considered the most serious of plant diseases, since it is widespread, it persists for many years in the soil and has a very wide host range (Masirevic and Gulya, 1992). *Sclerotinia* secretes toxins, including oxalic acid (Callahan and Rowe, 1991) which, acting as a toxin, causes pH variations, stem lesions and complete and irreversible plant wilting (Marciano *et al*., 1983; Bazzalo *et al.*, 1991). Oxalic acid concentrations in infected wilted leaves are higher than those in the leaves of uncontaminated plants (Noyes and Hancock, 1981) and the utilisation of culture filtrates of *Sclerotinia* in an attempt to screen tolerant genotypes in different plant species, including sunflower, has been suggested (Huang and Dorrel, 1978; Rowe, 1993). Recent studies by Tahmasebi Enferadi *et al*. (1998a; 1998b) have demonstrated that the exposure of sunflower to the toxic metabolites of *Sclerotinia* can result in plant wilting, tissue injury, an increase in the plant tissue levels of oxalic acid and shikimate dehydrogenase (SKDH) activity and synthesis of new proteins. The increase in SKDH activity is important because of its role in the biosynthesis of shikimic acid which is involved in the synthesis of lignin for cell walls (Buiatti, 1993; Carrera and Poverene, 1995). For the above reasons, in order to validate the *Sclerotinia* culture filtrate method as a screening strategy, in this work nine sunflower genotypes were artificially inoculated with fungus sclerotia and subjected to culture filtrate and their growth parameters and biochemical responses studied and compared.

Material and Methods

Sunflower Genotypes and Sclerotinia *Culture Filtrate Preparation* The genotypes used in this investigation were the inbred lines AC 2221, C, AC 4122, 7 ro, 28R and R978 obtained from the Crop Production Department of the University of Udine; the inbred line HA89 obtained from the USDA/ARS, Fargo; the high oleic hybrid Tenor, from AGROSEM and Cina2, an open pollinated population from China and tolerant to Sclerotinia head root .

The *Sclerotinia* culture filtrate was obtained starting from some black sclerotia collected from stems of infected plants and following the methodology described by Tahmasebi Enferadi *et al*. (1998a).

Culture Filtrate Experiment After germination in Petri dishes, at the two true-leaf stage (approximately 3 weeks old), the seedlings were transferred and grown in hydroponic culture, using Hoagland solution, at a temperature of 20-25°C, a relative humidity of about 60% and a light intensity of about 500 mE m-2 s-1. At least 4 week-old seedlings of each genotype were placed, for 24 h, in a 250 ml beaker glass containing *Sclerotinia* culture filtrate (75% v/v) and incubated at room temperature following the method suggested by Huang and Dorrel (1978) and modified by Tahmasebi Enferadi *et al*. (1998a).

The treatment consisted of the nine genotypes subjected to culture filtrate (with an oxalic acid concentration of about 0.46 g/l) while the controls consisted of plants grown in hydroponic culture throughout the entire period of the experiment. The treated genotypes and their controls were analysed 5 days after the treatment and the parameters considered were as follows :plant height (cm), stem diameter at soil level (mm), leaf area per plant (mm^2), total dry matter per plant (g), oxalic acid content of the upper leaves (mmol g^{-1}dry weight), enzyme activity E.U. (mmol sub min^{-1}mg $protein^{-1}$).

The experiment was carried out following a complete randomised block design with four replicates (beaker glass) and with four plants per replication. In order to eliminate differences between genotypes with different seedling vigour, many of the above parameters were expressed as a percentage of the control. An arc-sin square root transformation was then made on these percentages. Data were subjected to analysis of variance, with an appropriate ANOVA model.

Experiment with Myceliuml Inoculation The greenhouse test was conducted at the University of Udine, with the daytime temperature maintained at about 22°C and the night temperature at 18°C, with a relative humidity of 60 and 70% respectively. The plants were grown in sandy loam soil in 25 cm diameter pots, with 2 plants per pot. The inoculation was carried out on 43-days-old plants, following the method suggested by Cassels and Walsh (1995), by positioning pre-germinated sclerotia (1 mm of mycelium) from 7-days-old fungal cultures over the scraped area of the basal stem, covered with moist cotton wool and soil, to maintain humidity.32 plants (16 pots) per genotype were inoculated while other 16 plants (8 pots) were utilised as controls. The 32 inoculated plants were used to calculate the disease severity index (DSI) = (number of infected plants) x (severity class) / (number of inoculated plants). Severity class: 0= no disease symptoms, 5= plants killed. Ten days after the inoculation, the same parameters of the above experiment were analysed in the inoculated and control plants for each genotype, utilising the same experimental scheme except for the replication number, which in this experiment was three (pots) randomly chosen.

Determination of Oxalic Acid in Leaves and Shikimate Dehydrogenase (SKDH) Activity Assays Both oxalic acid content and the Shikimate activity in sunflower extracts was determined as previously described by Tahmasebi Enferadi et al., (1998b).

Results and Discussion

Symptoms on Growth Parameters Both treatments, namely the inoculation and the culture filtrate, caused an evident reduction in plant dry matter and leaf area, compared with the controls and, in particular, the culture filtrate showed a stronger effect than the artificial infection (Figure 1). Significant differences were recorded between genotypes and in particular AC 4122 and R 28 showed significantly lower reductions than the control and those observed in the other genotypes, in both treatments. The above two characters were also significant and negatively correlated with the disease severity index (DSI, data not shown) in both treatments, thus demonstrating the possibility of their being utilised as screening parameters.

Both treatments induced a severe necrotic reaction at the basal stalk level with a strong reduction of the stem especially in the culture filtrate treatment. However genotypes AC4122 and R28 had stem diameters much closer to those of their controls than the other genotypes (Fig.1) in response to the inoculation. This response can be explained as a reaction to the pathogen infection; an enhanced growth and intense lignification of the tissue at the basal level of the stem was observed, as previously obtained by Orellana (1975). Plant height was similarly reduced by both treatments, without any significant differences between genotypes.

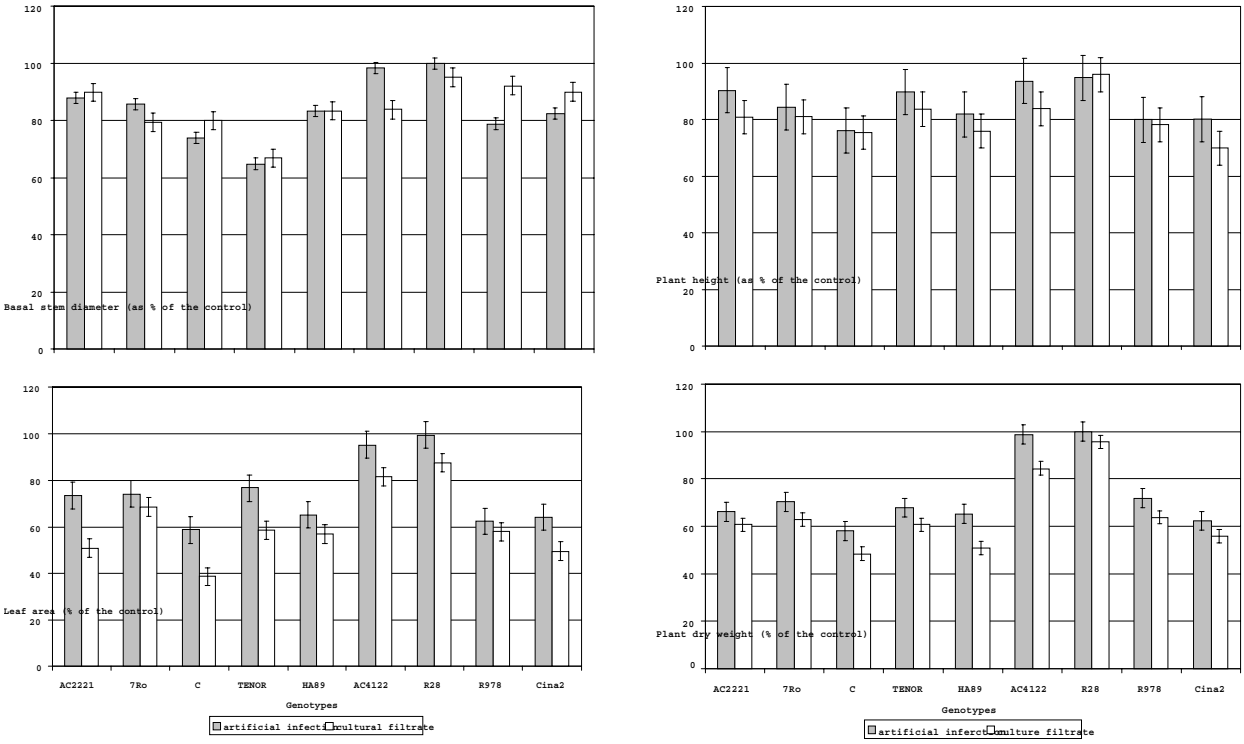

Figure 1 – Growth parameters of the genotypes analysed after artificial inoculation and exposure to culture filtrate. Values expressed as % of the controls. Bars represent L.S.D. for $P \leq 0.05$.

DSI, Oxalic Acid Content and SKDH Enzymatic Activity The disease severity index (DSI), used to express relative disease reaction in the artificially inoculated experiment , had significantly lower values for R28 (0.37) and AC4122 (0.22) than the other genotypes, demonstrating a tolerance to *Sclerotina* infection (Table 1).

Table 1 – Disease severity index (DSI) in inoculated plants and oxalic acid content and shikimate dehydrogenase (SKDH) enzymatic activity in leaves of sunflower plants subjected to artificial inoculation and to culture filtrate of *Sclerotinia*.

Genotypes	DSI	Oxalic acida in inoculated plants		Oxalic acida in plants in culture filtrate		SKDH activityb in inoculated plants		SKDHb activity in plants in culture filtrate	
		Control	Treated	Control	Treated	Control	Treated	Control	Treated
AC2221	0,75	288	528	552	1020	0,39	0,85	0,70	1,03
7Ro	0,78	312	648	504	1200	0,64	1,25	0,57	1,09
C	1,53	276	696	396	1008	0,49	1,57	0,39	1,03
TENOR	1,28	204	444	480	1176	0,49	1,33	0,82	1,14
HA89	1,00	324	612	432	888	0,50	1,35	1,21	1,65
AC4122	0,22	336	408	660	876	0,77	1,40	0,86	1,52
R28	0,37	300	372	840	1008	0,46	0,78	1,83	2,61
R978	0,66	264	444	636	1212	0,41	0,87	1,96	2,23
Cina2	0,80	300	576	480	912	0,52	1,10	0,66	1,75
L.S.D. for P £ 0.05	0,24	103	220	146	284	0,18	0,39	0,28	0,57

a expressed as (mmol g dry weight-1) b expressed as (mmol sub. min-1 mg protein –1)

The inoculation and the culture filtrate treatments led to a similar increment of oxalic acid content in the leaves (42.7 and 45.7%, respectively) in the treated plants with respect to their controls (Table 1). The lowest increments were observed in AC4122 (17.6% and 24.6%) and R28 (19.3% and 16.7%) in the inoculation and culture filtrate, respectively. This result

confirms previous observations by Buiatti, (1993),who reported that plant defence reactions can be induced by variations in the quantitative balances of certain substances, oxalic acid in the case of *Sclerotinia* infection, when they exceed a "threshold" concentration level.

The increases in oxalic acid levels obtained in the treated plants, agree with the results obtained by Noyes and Hancock (1981); although the values of oxalic acid they found in the treated plants (more than 100% higher than the controls) were much higher than in this experiment. This could be due to the different methods of oxalic acid detection used by the authors. The plants used as controls in the culture filtrate experiment had higher contents of oxalic acid than the control plants used in the inoculation (Table 1); this was due probably to the effect of the Hogland solution on the seedlings during the first development stages.

As shown in the Table 1, SKDH enzymatic activity clearly increased in the treated plants compared to the controls, in both experiments. In particular, the average increase of activity in the inoculated plants was 54.4%, with AC4122 and R28 having the lowest increment (41.0 and 45.0%, respectively). In plants subjected to culture filtrate the same mean increase was reduced to 38.2%. Figures 2 shows the positive and significant relationship between the increase of oxalic acid content detected in the leaves of treated plants in both experiments and the disease severity index (DSI). Figure 3 reports the positive significant relationship between the increase, compared to the controls, of SKDH activity in plants inoculated with the fungus and the DSI; in contrast, this relationship is completely lacking when the plants subjected to culture filtrate were considered.

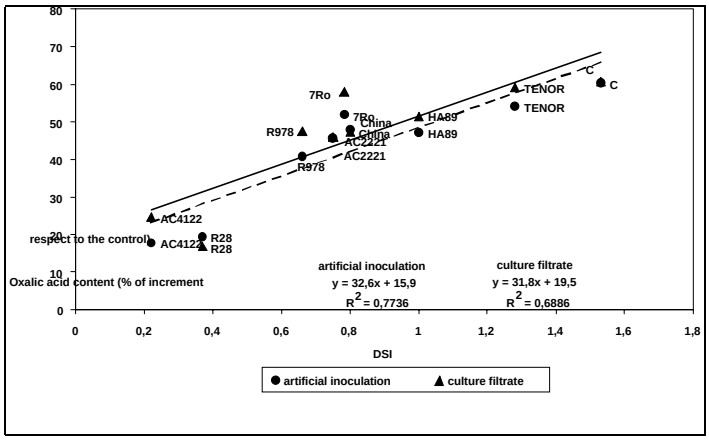

Figure 2 – Relationship among the disease severity index (DSI) and the increase of oxalic acid content in the plants subjetcd to artificial inoculation and to culture filtrate of *Sclerotinia*.

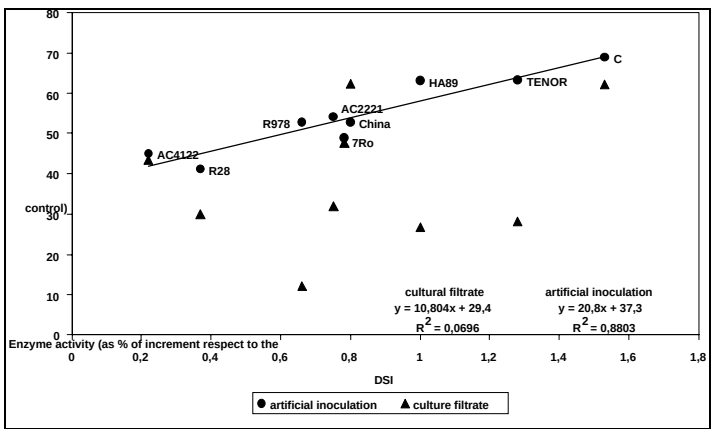

Figure 3- Relationship among the disease severity index (DSI) and the SKDH enzymatic activity in plants

Conclusions

The present studies show that the exposure of sunflower to toxic metabolites of *Sclerotinia* can result in plant wilting, tissue injury, development of lesions and an increase in the levels of oxalic acid and shikimate dehydrogenase activity. Of the genotypes tested, R28 and AC 4122 appeared to be the least affected by the pathogen and culture filtrate, thus confirming previous results (Tahmasebi Enferadi *et al*., 1998a) when the latter genotype appeared the most tolerant. The increase of oxalic acid in the plants obtained indifferently from plants subjected to both inoculation or to culture filtrate, can be successfully utilised to screen genotypes for tolerance to *Sclerotinia.* In contrast, the increased activity of SKDH in plants, seems to be triggered by a more specific reaction between mycelium and host and is not simply induced by the increase of oxalic acid in the tissue, in agreement with previous results obtained by Callahan and Rowe (1991). Even if the enzymatic activity was positively and significantly related to oxalic acid increases in the plants, further studies are required to explain the role of SKDH in the defence strategy of sunflower against *Sclerotinia*.

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