Study of the expression of shikimate dehydrogenase activity in sunflower genotypes treated with *Sclerotinia sclerotiorum*

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ABSTRACT

The expression of shikimate dehydrogenase in cotyledons of five sunflower inbred lines treated with *Sclerotinia sclerotiorum* was compared with exogenous application of synthetic oxalic acid. Normally, shikimate dehydrogenase becomes enzymatically active in sunflower at seed germination stage, and it reaches its maximum during the cotyledon stage, gradually decreases and disappears after four leaf stage. We found that shikimate dehydrogenase activity was very faint in control plant protein extract whereas its intensity greatly increased in samples derived from seedlings inoculated with *S. sclerotiorum* as well as with synthetic OXA at the same stage. The expression of shikimate dehydrogenase at the first phase of growth may serve as a tool for rapid screening and selection of resistant genotypes of sunflower to *S. sclerotiorum.* Some agronomy parameters in terms of plant dry and fresh weight and the total chlorophyll concentration were assessed for both treatments compared with their untreated controls. Exogenous oxalic acid treatment caused more deleterious effects in comparison with its endogenous production of the pathogen, considering stem rot and eliciting photosynthesis reduction. The excessive toxicity of exogenous treatment suggests that *S. sclerotiorum* infection triggers a more complex metabolic pathway involvings oxalic acid secreted by the pathogen.

Key words: dehydrogenase activity – *Helianthus annuus* – resistant genotypes – *Sclerotinia sclerotiorum* – screening – shikimate.

INTRODUCTION

Sclerotinia root, stem, and head rot are major diseases of sunflower caused by the pertotrophic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Rönicke et al., 2005). The general inability of economically important crops to develop germplasm resistant to this pathogen has focused attention on the need for a more detailed understanding of the pathogenic factors involved in disease development (Cessna et al., 2000). Oxalic acid secretion by *Sclerotinia* appears to be an essential determinant of its pathogenicity (Maxwell and Lumsden, 1970; Noyes and Hancock, 1981; Marciano et al., 1983; Godoy et al., 1990; Dutton and Evans, 1996; Zhou and Boland, 1999). Evidence for such involvement is based on the recovery of millimolar concentrations of oxalate from infected tissues (Bateman and Beer, 1965; Maxwell and Lumsden, 1970; Marciano et al., 1983; Godoy et al., 1990) and from the manual injection of oxalate, or of culture filtrate containing oxalate, into plants and observation of the development of *Sclerotinia* disease-like symptoms independent of the pathogen (Bateman and Beer, 1965; Noyes and Hancock, 1981).

Speculation regarding the mechanism or mechanisms by which oxalate secretion might enhance *Sclerotinia* virulence currently centers on three modes of action (reviewed in Dutton and Evans, 1996). First, because several of the fungal enzymes secreted during invasion of plant tissues (e.g., polygalacturonase) have maximal activities at low pH, various researchers have postulated that oxalate might aid *Sclerotinia* virulence by shifting the apoplastic pH to a value better suited for enzymatic degradation of plant cell walls (Bateman and Beer, 1965). Second, because oxalate may be directly toxic to host plants, presumably because of its acidity, the secretion of oxalate has been suggested to weaken the plant, thereby facilitating invasion (Noyes and Hancock, 1981). Finally, chelation of cell wall Ca^{2+} by the oxalate anion has been proposed both to compromise the function of Ca^{2+}-dependent defense responses and to weaken the plant cell wall (Bateman and Beer, 1965). Although each of these hypotheses has its logical appeal, evidence supporting them is incomplete, and arguments against their validity have also been made (Dutton and Evans, 1996).

Shikimate dehydrogenase (EC 1.1.1.25) (SKDH) is an important biochemical marker produced by plants for investigation of *S. sclerotiorum* infection that catalyzes the fourth step in the shikimate pathway, which is essential for biosynthesis of aromatic amino acids and aromatic compounds. The increase in SKDH activity occurring after *Sclerotinia* infection affects the biosynthesis of shikimic acid, which is involved in the synthesis of lignin for cell walls (Buiatti, 1993; Carrera and Poverene, 1995) and is considered to be the most interesting component in relation to the plant resistance to *S. sclerotiorum* (Quillet, 1990).

The aim of this study was to determine the effects of OXA treatment in *Helianthus annuus* L. either when OXA was endogenously produced by *S. sclerotiorum* or, alternatively, when it was exogenously treated as synthetic moiety. Study of the expression of SKDH activity may help to develop a fast and reliable screening technique in breeding sunflowers for resistance to *S. sclerotiorum*.

MATERIALS AND METHODS

Fungal and plant material

Black sclerotia of *S. sclerotiorum* collected from stems of infected plants were germinated and grown on potato dextrose agar (PDA) at 25 °C. After several passages on PDA and controlling the proper hyphae by observation under optical microscope 400 X, sclerotia were subcultured on PDA (Becton Dickinson, Sparks, MD, USA) under light (24 h/day). After 3 days, 0.2 cm agar plugs were removed with a sterile cork borer from the leading edge of the mycelia and were subcultured on PDA agar plate, 0.5 cm agar plugs were removed from the leading edge of the second two-day old mycelia and used for inoculation.

Five inbred lines of sunflower of different origins were used in the experiments: AC 4122 and C are maintainer inbred lines, developed at University of Udine from an Italian open pollinated population ALA, HA 89 is a maintainer inbred line and HA 410 (Reg. no. GP-227) and HA 411 (Reg.no.GP-228) are inbred lines released by USDA-ARS, Fargo, ND, North Dakota. AC 4122 and HA410 are resistant inbred lines, C and HA 89 are susceptible inbred lines, and HA 411 is an intermediate inbred line. Seeds of five genotypes were surface sterilized as described by Burrus et al. (1991) and germinated in sterile test tubes (130 x 25 mm) on a solid MS medium (Murashige and Skoog, 1962).

Plugs of PDA prepared as described earlier were placed on the leaves which were wounded slightly. Leaves of uninoculated, control plants were treated similarly with PDA agar plugs without the mycelia. The inoculated parts of plants were then washed by sterilized water and transferred to Hoagland solution (H2395, Sigma Chemical Co. prepared according to manufacture's directions, autoclaved and stored at room temperature) and maintained at 20-25°C, relative humidity about 40-50% and light intensity about 500 mM. m^{-2} s^{-1}.

Preparation of synthetic oxalic acid

A stock solution of 1 M oxalic acid (Sigma Chemical Co.) was prepared, then it was diluted to obtain the same toxin concentration of culture filtrate (toxin concentration was estimated using Oxalate kit 591C followed by spectrophotometer assessment).

The recovery of vegetal extraction

Five days after exposure to *S. sclerotiorum* and synthetic OXA, the plant tissue above the cotyledons (also from untreated control plants) was collected and homogenized in a mortar and placed in a sealed tube containing buffer 50 mM Tris-HCl, (pH 7.4), 0.25 M sucrose, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM PMSF (phenylmethanesulfonyl fluoride), 2.5% v/v β-mercaptoethanol. After homogenization and centrifugation at 2000 *g for 5 min, the supernatant was used for OXA determination, total protein determination, and SKDH activity assay.

Oxalic acid measurement

Ten µl of extracted plant material as previously described was used for measuring toxin (OXA) concentration by oxalate kit 591 C followed by spectrophotometer assessment at 590 nm wave lengths.

Determination of total soluble protein and Shikimate dehydrogenase activity assays

The extracted plant material was employed to determine the total protein content, using the Bio-Rad protein assay kit with BSA as standard (Bradford, 1976) followed by spectrophotometer assessment at 595 nm wave length.

Native-PAGE

Native Polyacrylamide Gel Electrophoresis was performed using 12% (W/V) polyacrylamide slab gel in 0.2 M Tris, 2 mM EDTA and 0.15 M boric acid (pH 8.5) as electrode buffer (Guries and Ledig, 1978). Staining of SKDH was done by fixing for an hour in buffer solution containing tetrazolium salt as described by Tanksley and Rick (1980).

Experimental design and statistical analysis

The treatments corresponded to five genotypes inoculated with *S. sclerotiorum* culture filtrate, treated with synthetic OXA and the controls, which were grown in hydroponics with Hoagland solution under similar conditions. The analyses of total fresh weight, total dry matter per plant at the end of the experiment, OXA concentration (mM), and chlorophyll concentration (mg m^{-2}) 5 days after treating and the expression of SKDH were carried 48 h after treating with either *S. sclerotiorum* or exogenous OXA.

The experiment was carried out following a bifactorial completely randomized block design with three replicates and four plants for each replication. The first factor, genotype, was constituted by the five inbred lines, and the second factor, toxin treatment, was constituted by endogenous OXA produced by *S. sclerotiorum* and exogenously applied synthetic OXA. Statistical analyses of triplicate determinations of OXA contents and enzymatic activity of SKDH from five genotypes were subjected to Analysis of Variance. Significant differences were expressed as $P<0.01$, and the least significant difference procedure was used to compare means of treatments. Correlation coefficients and regression analysis were calculated between the variables having significant differences between genotype means.

RESULTS AND DISCUSSION

The effects of two treatments, inoculation with *S. sclerotiorum* and exogenous oxalic acid, on plant growth were compared by measuring fresh weight and dry weight (plants were dried at 60 ºC in an oven for 3 days), which were the only growth parameters that could be calculated in early growth phase. Significant differences were recorded between genotypes for dry and fresh weight. In samples treated with culture filtrate, the fresh weights (considered as a percentage of controls) were significantly higher in HA 410, intermediate in HA 411 and AC 4122, and low in C and HA 89. These differences revealed an individual variability of responses to toxin penetrated into the cells, which confirms the polygenic nature of this disease (Mestries et al., 1998). The dry matter of these samples did not show significant differences between genotypes (Table 1). These data indirectly suggest that *S. sclerotiorum* manipulates the metabolism of host-derived carbohydrates and consequently increases in cell water content.

In the cases of samples treated with synthetic OXA, fresh weight of HA 411, AC 4122 and C had higher values whereas other genotypes followed them with lesser significant differences. Concerning dry matter, there were no significant differences except in resistant line AC 4122 with the lowest dry weight. These results provide an alternative explanation for oxalate-induced wilting. It seems that synthetic OXA induces an equal effect of destruction on all resistant or susceptible genotypes. This firstly causes a great reduction in plant growth, then self-reconstruction of the plant happens and it continues its growth.

Table 1. Growth characters of five sunflower genotypes analysed 5 days after inoculation with *S. sclerotiorum* and treated with synthetic OXA

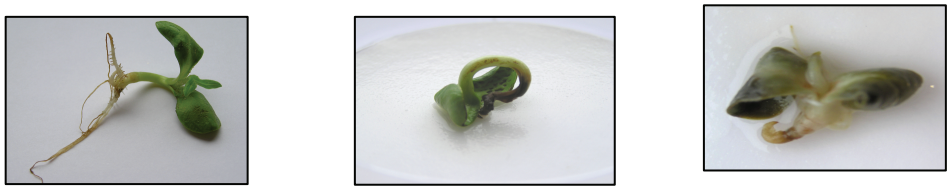
Genotypes	Synthetic OXA		*S. sclerotiorum* inoculation	
	Dry Weight Plant (%)[1]	Fresh Plant Weight (%)[1]	Dry Weight Plant (%)[1]	Fresh Plant Weight (%)[1]
AC 4122	58.6 b[2]	43.91 a	65.6 a	36.8 b
C	76.4 a	39.3 ab	64.9 a	27.7 c
HA 89	71.1 a	30.9 c	64.4 a	28.6 c
HA 410	71.1 a	36.9 bc	71.4 b	47.8 a
HA 411	75.5 a	43.4 a	68.4 ab	36.2 b

[1]Values are reported as percentage of the controls,

[2]Means followed by the same letter are not significantly different at 1% level as indicated by Duncan's Multiple Range Test.

The toxic metabolite of pathogen causes a decrease in chlorophyll (chl) concentration and this reduction is clearly associated with other symptoms of phytopathogenicity, i.e. stem rot. Chl

concentration data will provide information on a plant's photosynthetic potential (Raymond et al., 2004). The effect of both treatments on plant metabolism was revealed as a reduction in Chl concentration (data not shown). This phenomenon for the samples treated with synthetic OXA was not accompanied by any signs of stem rot and basal stalk rot, which implies the different nature and effect of OXA on the plant (Fig 1).



a **b** **c**

Fig. 1. Comparison of growth in plants a) uninoculated control, b) inoculated with *S. sclerotiorum* and c) treated with synthetic OXA.

The intracellular OXA content in sunflower seedlings was determined five days after inoculation with *S. sclerotiorum* or synthetic oxalic acid treatment to verify whether the metabolic response to OXA could be correlated with disease resistance.

As reported in Fig. 2, in uninoculated lines C (1.10 mM) and HA 89 (1.21 mM) a higher OXA content was observed, which demonstrates that they are more susceptible to fungal disease as compared to the other lines. This confirms previous observations by Tahmasebi Enferadi et al. (1998 b) about the different thresholds of OXA concentration between different genotypes.

Concerning samples inoculated with *S. sclerotiorum*, OXA concentration values were the highest in susceptible HA 89 (1.81 mM) and C (2.6 mM) when compared to their untreated controls. On the contrary, it was observed that OXA intracellular concentration in samples treated with synthetic acid was lowest in HA 89 (2 mM) whereas AC 4122 had the highest content.

Since OXA concentration increases in pathogen-infected plants, our data demonstrate that the more resistant the plants, the more they were able to control catabolism of this acid, as shown in HA 410 and AC 4122. This is probably due to an intercellular mechanism which inhibits abnormal increases in their pH. Therefore, specific macromolecules are produced by a pathogen that can be recognized by the plant (Buiatti, 1993) and lead to the activation of a host defense response. These signals were absent after synthetic OXA treatment, causing the plant to be unable to manage OXA.

Other studies showed that phenolic compounds play an important role in plant defense responses against pest and pathogens (Nicholson and Hammerschmidt, 1992). In sunflower the induction and accumulation of phenolic compounds, their deposition on cell walls and lignification is a well-characterized mechanism of disease resistance against *S. sclerotiorum* (Prats et al., 2003). A higher content of phenolic compounds in resistant varieties was observed as compared to susceptible ones (Prats et al., 2003; Rodríguez et al., 2004). Conceivably, resistant plants had higher activity levels of phenylalanine ammonia–lyase (PAL), which provides the biosynthesis of important phenolic derivatives such as lignin.

Similar to phenolic compounds and PAL, shikimic acid and the related enzymatic activity of SKDH are used in order to find out a biochemical paradigm, which provides a clear correlation with disease resistant genotypes. SKDH is an intermediate step in aromatic amino acid biosynthetic pathway, essential to lignin production, and is considered as a resistance mechanism against *S. sclerotiorum* related to its chemical and/or physical cell barriers.

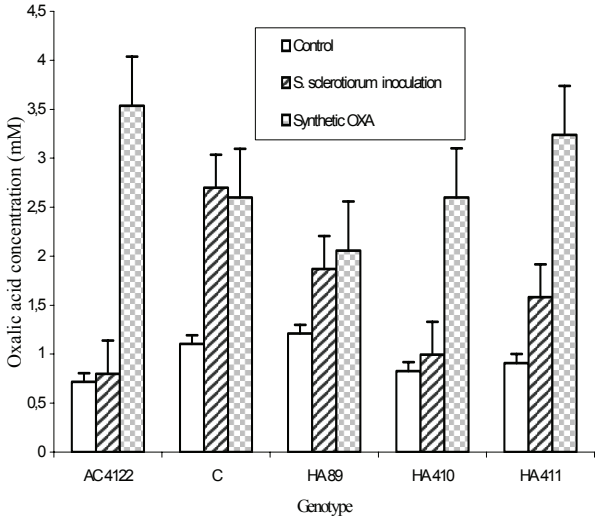


Fig. 2. OXA concentration of different sunflower genotypes 5 days after inoculation with *S. sclerotiorum* and synthetic OXA and untreated plants (control). Bars represent L.S.D. for $P < 0.01$

SKDH that becomes enzymatically active in sunflower at seed germination stage, and it reaches its maximum during the cotyledon stage, gradually decreases and stops after 4-leaf stage as reported by Diaz et al. (1997). SKDH reactivates treating the plant either with pathogen or synthetic OXA. Parental inbred lines have a single band with identical mobility (Carrera and Poverene, 1995), indicating the presence of the same allele in all genotypes, as reported by Ledoux (1992). Enzyme SKDH has a monomeric structure, encoded by a single gene and a single locus with two different co-dominant alleles in heterozygous plants, *skdh*-a and *skdh*-b, with the molecular weight of 64.5 kDa and 58.9 kDa, respectively (Tahmasebi et al., 1998a). The increase in SKDH activity for both homozygous and heterozygous individuals following the attack of *S. sclerotiorum* is accompanied by the expression of only skdh-b. The lack of the expression of skdh-a in homozygous individuals confirms the hypothesis by which skdh-a is considered a null allele, as described by Goodman et al. (1980). Both alleles have most likely the same domains with a few changes in the variable regions, which concerns regions interacting with OXA. In Fig. 3, the domain family of SKDH is shown. It is suggested that the interaction between OXA and reactivation of skdh-b relates to the third domain, Shikomate_DH. This domain involves the biosynthesis of aromatic amino acids and is related to the mechanism of resistance. It seems that other domains have a structural role.

Fig. 3. Domain pattern of SKDH along its polypeptide

A study of the expression of SKDH on Native-PAGE 48 h after treatment of the studied inbred lines demonstrated that only a single allele, *skdh*-b, expressed and its mobility was identified. Conceivably, to data dealing with the enzymatic activity dosage, SKDH was very faint in control plant protein extract whereas its intensity greatly increased in samples derived from seedlings inoculated with *S. sclerotiorum* as well as with synthetic OXA (Fig 4.)

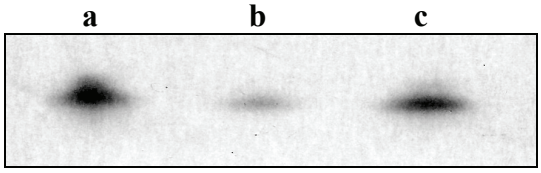


Fig. 4. Expression of skdh-b at the end of cotyledons in homozygous lines of sunflower on Native-PAGE 48 h after treating with a) Synthetic OXA, b) untreated plant, control, and c) *S. sclerotiorum* inoculation.

CONCLUSIONS

In conclusion, the differences observed between symptoms generated by OXA produced by pathogen and OXA originating from a synthetic source can be related to the different nature of biochemical pathway elicited by each treatment, both in resistant and in susceptible inbred lines. Subsequently, this prevents the use of synthetic OXA instead of direct inoculation of plants in rapid screening methods for identification of genotypes resistant to *S. sclerotiorum*. Other advantages of measuring SKDH activity as a rapid and reliable method of screening, are the early discrimination of resistant genotypes in the first growth stage, at the laboratory, on many individuals (since *S. sclerotiorum* is of a polygenic nature, it needs to provide a resistant mass individual) and its cost effectiveness.

Furthermore, our results indicate that SKDH may be a promising biochemical marker that could be used in breeding programs to discriminate between sunflower genotypes resistant and susceptible to *Sclerotinia* infection.

Although different disease resistance mechanisms can be activated simultaneously during defense response, SKDH levels could be directly evaluated to identify resistant lines or, possibly, related to other molecular markers such as total content of phenolic compounds and PAL activity.

ACKNOWLEDGEMENTS

We thank Azienda agraria universitaria "A. Servadei" for providing us with the genetic materials. This work was supported by grants from the Regione Autonome Friuli Venezia Giulia on the improvement of the Project Cargi2.

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