SHIKIMATE DEHYDROGENASE ACTIVITY AS AN INDICATOR OF THE SUNFLOWER PLANT RESISTANCE TO BASAL STALK ROT (*SCLEROTINIA SCLEROTIORUM)*

Sattar Tahmasebi Enferadi, Gianpaolo Vannozzi, Zohreh Rabiei, Dipartimento di Scienze Agrarie e ambientali Università degli Studi di Udine, via delle Scienze 208, 33100 Udine, Italy
E-mail: thamasebi@uniud.it
E-mail: gianpaolo.vannozzi@uniud.it
E-mail: z_rabiee@yahoo.com

Ahmad Ali Attary, Seed Plant Improvement Institute (SPII) Box: 31746/117 Karaj, Iran
E-mail: spii.int@abdnet.com

Abstract

Greater amounts of oxalic acid and high shikimate dehydrogenase (SKDH) activity are associated with *Helianthus annuus* L. infected with *Sclerotinia sclerotiorum* (Lib.) de Bary, which causes Sclerotinia crown and stem rot. In order to evaluate the relationship between these two aspects of the infection process, we sampled seedlings of five sunflower genotypes, all inbred lines (HA 410, HA 411, HA 89, C, AC4122) which were subjected to artificial inoculation with the culture filtrate of *Sclerotinia sclerotiorum.* We also compared the effect of exogenous synthetic and endogenous production of oxalic acid in the samples. The reaction of the genotypes was measured as the reduction in many growth characters compared with the control. The measurements revealed more deleterious effects of exogenous oxalic acid than its endogenous addition, probably due to some unknown complex metabolism of this acid secreted by the pathogen. A positive correlation was found between the increase of oxalic acid and the increase of shikimate dehydrogenase activity in both treatments.

Introduction

Sclerotinia sclerotiorum (Lib.) de Bary, an aggressive fungal pathogen, causes infection in hundreds of plant species worldwide, including crop plants such as soybean, bean, sunflower, and canola (Purdy, 1979). The development of the disease in the plant is related to high polygalacturonase activities (Bazzalo et al., 1991), the enzymatic degradation of the cell wall membrane in the host cells (Lumsden, 1979) white rot, the production of oxalic acid which, acting as a toxin, causes pH variations, stem lesions and complete and irreversible plant wilting (Noyes and Hancock, 1981; Marciano et al., 1983). Some studies indicated that oxalic acid was partially responsible for pathogenesis (Marciano et al., 1983). Cotton et al. (2003)

showed that progressive acidification of the ambient medium by the fungus is a major strategy for the sequential expression of pathogenicity factors. In certain phytopathogenic species of fungi, oxalic acid accelerates plant tissue maceration through synergism with polygalacturonase (Cotton et al., 2003). Oxalic acid concentration in infected wilted leaves is up to 15 times higher than in leaves of uncontaminated plants and often the concentration of this acid in the tissues is used to measure the virulence of the fungus (Noyes and Hancock, 1981).

Shikimate dehydrogenase (EC 1.1.1.25) is one of the more important biochemical markers in the investigation of *Sclerotinia sclerotiorum;* it catalyses the fourth step in the shikimate pathway, which is essential for biosynthesis of aromatic amino acids and aromatic compounds. 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), and is considered to be the most interesting in relation to resistance to Sclerotinia (Quillet, 1990), in fact, its pathway leads to the biosynthesis of phytoalexins, antimicrobial compounds produced by the infected host plant to inhibit fungal development (Bert et al., 1993).

Genetic resistance is considered to be the most effective control measure, and there are some potential sources of tolerance to Sclerotinia, but no sunflower genotype with absolute resistance to Ssclerotinia has yet been found (Masirevic and Gulya, 1992). That may be due to 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 (Tahmasebi Enferadi et al., 1998a). The objectives of this study were to determine what deleterious effects fungal exudates of culture filtrates of *Sclerotinia* have on different sunflower genotypes, and to attempt to measure the SKDH activity, which according to the author's earlier work (Tahmasebi Enferadi et al., 1998b) implied an involvement of this enzyme in the defence mechanisms, and finally quantifying the oxalic acid in two situations of exogenous and endogenous oxalic acid addition to plants.

Materials and Methods

Plant Material. Five genotypes of different origin were used in the experiment: AC 4122 and C, maintainer inbred lines, bred by Udine University from an Italian open pollinated population (ALA); HA 89 maintainer inbred line, and HA 410 (Reg. no. GP-227, and HA 411 (Reg. no. GP-228), inbred lines released by USDA-ARS in North Dakota.

Preparation of Sclerotinia sclerotiorum Culture Filtrate. Some black sclerotia of *Sclerotinia*, collected from stems of infected plants, were maintained on potato dextrose agar (PDA) at a temperature of 25C. After 14 days plates were transferred to Erlenmeyer flasks containing 1000 ml of liquid medium (DIFCO®AND BBL® CZAPEK-DOX without agar) at a constant temperature of 27C. After 40 days, the culture solution was filtered. Toxin (oxalic acid) concentration was calculated using an (Endpoint enzymatic-colorimetric method Endpoint Sigma, 591C kit, Sigma Chemical Co. St. Louis, MO, USA). Oxalic acid was used as the standard.

Plant Growth Method and Inoculation Test. The seeds of five studied genotypes were sterilized as described by Burrus et al. (1991) and were germinated in sterile test tubes (130 x 25 mm) on a solid MS medium (Murashige and Skoog, 1962). At the initial stage of B1, the

first treatment including seedlings of each genotype were placed in a 250-ml vial containing 250 ml *Sclerotinia* culture filtrate and incubated at room temperature at pH=4 for 24 hr as described by Tahmasebi Enferadi et al. 1998a) and Huang and Dorrel (1978). The second treatment consisted of the five genotypes subjected to synthetic oxalic acid at the same concentration, pH and time as the first treatment, then the roots of the plants were washed and all samples transferred to Hoagland's solution at a temperature of 20-25C, relative humidity of about 40-50% and light intensity about 500 mM/m2/s. Controls consisted of plants for each genotype grown in sterile test tubes on a solid MS medium during all the time of the experiment.

Extraction of Soluble Protein Fraction from Leaves. The upper cotyledon tissue was homogenized in a mortar and placed in a sealed tube containing buffer 50 mM Tris-HCl, pH 7.4, 0.25 M saccharose, 1mM EDTA, 1mM PMFS; 2.5% (v/v) β-mercaptoethanol. After homogenization and centrifugati0n at 500 rpm for 5 min, the supernatant was used for oxalic acid determination as previously described. 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 (Bradford, 1976).

Shikimate Dehydrogenase (SKDH) Activity Assays. Shikimate activity in sunflower protein extracts was colorimetrically determined using the Pearse (1972) and Glenner (1977) method. 100μl of plant extract was taken and incubated at 37C for 30 min in a buffer solution as described by Tanksley and Rick (1980), giving a final volume of 1 ml. The assay mixture (total volume 1 ml) contained 0.1 M Tris-HCl, pH 8.5, 0.01 mM MTT and 2.5% (v/v) β-mercaptoethanol, and was incubated for 1 min at a temperature of 37C (21)/7/. During the reaction, tetrazolium salt was produced, thus leading to a change in solution absorbance at 570 nm as reported by Ponce et al. (1994).

Experimental Design and Statistical Analysis. The treatments corresponded to five genotypes subjected to the culture filtrate, the treatments subjected to synthetic oxalic acid, and the controls, all grown in the same period and following the same experimental design. The treated genotypes and their controls were analysed two times: before treatment and 10 days after the treatment. The parameters considered were total fresh weight, total dry matter per plant (at the end of the experiment) (g), the oxalic acid concentration (mM), and enzymatic activity E.U. (μmol sub mg/ protein/ min).

The experiment was carried out following a complete randomised block design with three replicates with four plants for each 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. We conducted statistical analyses of triplicate determinations of oxalic acid content and enzymatic activity of SKDH from the five genotypes considered as described by Tahmasebi Enferadi et al. (1998b) by ANOVA. Significant differences were expressed as $P<0.01$, and the least significant difference procedure was used to compare means of genotypes. Correlation coefficients and regression analysis were calculated between the variables with significant differences between genotype means.

Results and Discussion

The significant differences between genotypes and all three treatments (control, culture filtrate, and synthetic OXA) from the aspect of total fresh weight and total dry matter were recorded. There was also a significant interaction between genotypes and treatments.

The values of fresh weight as a percentage of the controls in HA 410 are significantly higher in the case of samples treated by culture filtrate, compared to other genotypes, while two other genotypes, HA 411 and AC 4122, had intermediate values, and genotype C and HA 89 had the lowest values. That revealed different plant responses to toxin penetrating into the cells. The dry matter of these samples didn't show significant differences between genotypes.

As reported in Table 1, genotypes HA 411, AC 4122 and C had the higher values, whereas other genotypes followed them with low significant differences; and for dry matter, there was no significant difference except AC 4122 had lower weight.

Table 1. Growth characters of genotypes analysed 10 days after exposure to toxic metabolites of *Sclerotinia sclerotiorum* and treated by synthetic oxalic acid.

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

Means followed by the same letter are not significantly different at the 5% level as indicated by Duncan's Multiple Range Test.

[a] Values are reported as percentage of the controls.

There was a significant difference between genotypes and treatments in oxalic acid concentration; moreover their interaction was also significantly different.

The difference of oxalic acid in controls between HA 411 and HA 89 was significant. Line C (2.58 mM) and HA 89 (1.44 mM) are susceptible and had higher oxalic acid content, which confirms previous observation by Tahmasebi Enferadi et al. (1998 b). The fluctuating oxalic acid content in HA 89 and C with the lowest concentration in the controls and in HA 410, HA 411 and AC4122 were irregular and regular, respectively.

The oxalic acid concentration among samples treated by culture filtrate was the highest in HA 89 (1.81 mM) and had significant differences from HA 410 and HA 411.

Oxalic acid content of samples treated by synthetic OXA in HA 89 was the lower (2.6 mM), whereas AC4122 had the highest content (Figure 1). Oxalic acid concentration in diseased plants increased and the more resistant the plant, the more control of anabolism of this acid, probably as a result of the intercellular mechanism of the plant providing an abnormal increase in pH. The increase of oxalic acid content in all genotypes of samples treated by synthetic OXA was higher than of samples treated by culture filtrate. That revealed the increment of oxalic acid doesn't act in a defence mechanism alone; it follows a complex mechanism.
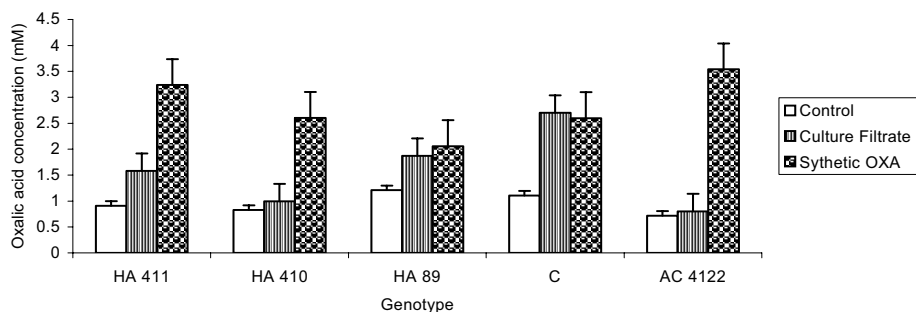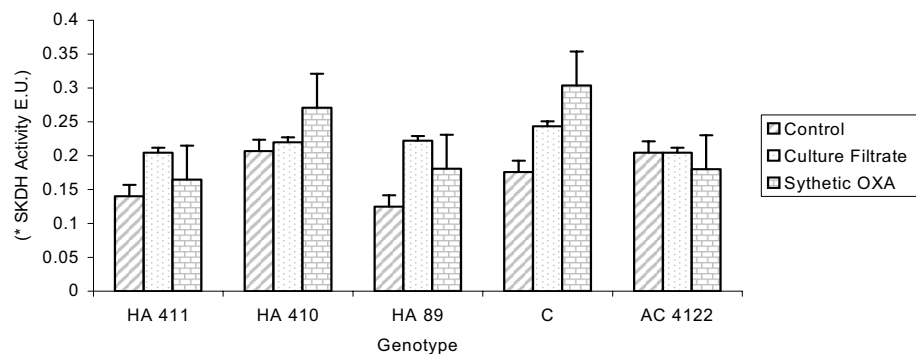
Figure 1. Oxalic acid concentration in control plants10 days after exposure to toxic metabolites of *Sclerotinia sclerotiorum* and synthetic OXA. Bars represent LSD. for P < 0.01.

*SKDH Activity E.U. (μmol sub μg/protein/min)

Figure 2. SKDH activity in control plants 10 days after exposure to toxic metabolites of *Sclerotinia sclerotiorum* and synthetic OXA. Bars represent L.S.D. for P < 0.01.

Data for SKDH activity can be found in Figure 2. Significant differences of controls between the genotypes were identified, and controls were significantly lower than the other samples, except in AC4122. HA 410 (0.22 E.U.) and HA 89 (0.17 E.U.) had the highest and lowest enzymatic activity, respectively. In samples treated by culture filtrate, enzymatic activity of HA 411 increased greatly (0.24 E.U.) with respect to its control, whereas SKHD in samples treated by synthetic OXA decreased in all genotypes except HA410 and C, related to the samples treated by culture filtrate, due to the strange activity of SDKH against synthetic oxalic acid.

We found that the enzymatic activity was higher in resistant genotypes than susceptible. Ledoux (1992) reported that the enzyme SKDH becomes active in sunflower at seed germination, and has its highest activity during the cotyledonal stage, gradually decreasing and disappearing after the 4-leaf stage.
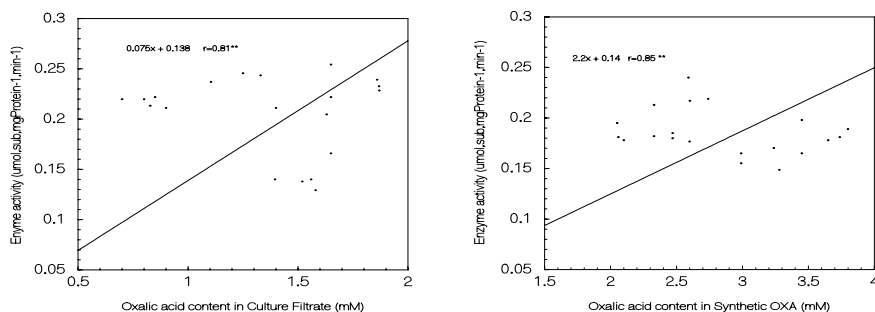
Figure 3. Relationship between oxalic acid concentration and enzymatic activity in the 5 genotypes 10 days after treatment.

There was an increase in both oxalic acid concentration and SKDH activity of samples treated by culture filtrate and synthetic OXA. A positive correlation (r=0.81 and r=0.85, respectively). Figure 3 indicates that the extra presence of oxalic acid which originated from the pathogen had a non-homogenous distribution in relation to the regression line, different from samples treated by synthetic OXA, which reveals the polygenic character of this disease.

Conclusions

We concluded that the differences between symptoms of disease caused by oxalic acid produced by the pathogen and synthetic oxalic acid was due to its different nature. Moreover oxalic acid in two types, natural and synthetic, has an important role in relation with the increase in SKDH enzymatic activity.

Acknowledgments

References

Bazzalo, M.E., P. Dimarco, F. Martinez and G.R. Daleo. 1991. Indicators of resistance of sunflower plant to basal stalk rot *Sclerotinia sclerotiorum*: Symptomatological, biochemical, anatomical and morphological characters of the host. Euphytica. 57:195-205.

Bradford, M.M. 1976. A rapid and sensitive method of quantitation of microgram quantities of protein utilizing the principle of protein binding. Anal. Biochem. 72:248-254.

Bret, E., F. Vear and D. Tourvielle. 1993. Utilisation des marqueurs moleculaires pour la resistance au Sclerotinia. Les Rencontres Annuelles du CETIOM. INRA., Centre Recherches Agronomiques Clermont-Ferrand, France, p 4.

Buiatti, M. 1993. Basi biochimiche della resistenza a patogeni e loro controllo genetico. In Crinò et al. (eds). Miglioramento genetico delle piante per la resistenza a patogeni e parassiti. Edagricole, Bologna, p. 654.

Burrus, M., C. Chanabe, G. Alibert, and B. Bidney. 1991. Regeneration of fertile plants from protoplasts of sunflower (*Helianthus annuus* L.). Plant Cell Rep. 10:161-166

Carrera, A., and M. Poverene 1995. Isozyme variation in *Helianthus petiolaris* and *H. annuus*. Euphytica, 81: 251-257.

Cotton, P., Z. Kasza, C. Bruel, and C. Rascle. 2003. Ambient pH controls the expression of endopolygalacturinase genes in the necrotrophic fungus *Sclerotinia sclerotiorum*. FEMS Microbiology Letters. 277:163-169

Glenner, G. 1977. Formazans and tetrazolium salts. In: R.D. Lillie (eds) H.J. Conn's Biological Stains. Williams and Wilkins Company, Baltimore, p. 225-235.

Huang, H.C., and D.G. Dorrel. 1978. Screening sunflower seedlings for resistance to toxic metabolites produced by *Sclerotinia sclerotiorum*. Can.J. Plant Sci. 58:1107-1110.

Ledoux, A. 1992. Utilization des marqueurs isoenzymatiques dans une selection genealogique chez le tournesol. Stage realizé à la Station d'Ameliorement des plantes de l'INRA de Clermond-Ferrand, p.30.

Lumsden, R.D. 1979. Histology and physiology of pathogenesis in plant diseases caused by Sclerotinia species. Phytopathology. 69:890-896

Marciano, P., P.D. Lenna, and P. Magro. 1983. Oxalic acid, cell wall-degrading enzymes and pH in pathogenesis and their significance in the virulence of two Sclerotinia sclerotiorum isolates on sunflower. Physiol. Plant Pathol. 22:339-345.

Masirevic, S., and T.J. Gulya. 1992. Sclerotinia and Phomopsis - two devastating sunflower pathogens. Field Crops Research. 30:271-300.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.

Noyes, R.D., and J.G. Hancock. 1981. Role of oxalic acid in the Sclerotinia wilt of sunflower. Physiol. Plant Pathol. 18:123-132

Pearse, A.G.E. 1972. Histochemistry. Theoretical and applied. Volume 2. Williams and Wilkins Company, Baltimore, 3rd edition, p. 760-1518.

Ponce, M.M., A.I. Navarro, G.M.N. Martìnez, and C.R. Alvares. 1994. Efecto antigiardiàsico in vitro de 14 extractos de plantas. Rev. Inv. Clin. 46:343-347.

Purdy, L.M., 1979. *Sclerotinia sclerotiorum*: history, diseases and symptomatology, host range, geographic distribution, and impact. Phytopathology. 69:875-880.

Quillet, M.C. 1990. Etude du polymorphisme enzymatique et proteique chez le tounesol, application à la charactérisation de ligneés. Stage realizé à la Station d'Ameliorement des plantes de l'INRA de Clermond-Ferrand, p.30.

Tahmasebi Enferadi, S., G.P. Vannozzi, D. Gomez-Sanchez, and M. Baldini. 1998a. Results of screening sunflower for resistance to toxic metabolites produced by *Sclerotinia sclerotiorum (*Lib) de Bary and prospects for the use of biochemical markers in breeding. Agr.Med. 128:47-58.

Tahmasebi Enferadi, S., D. Gomez-Sanchez, M. Baldini and G.P. Vannozzi. 1998b. Effect of *Sclerotinia sclerotiorum* (Lib.) de Bary culture filtrate on sunflower characters, oxalic acid content and shikimate dehydrogenase activity. Helia. 28:81-96.

Tanksley, S.D., and C.M. Rick. 1980. Isozymic gene linkage map of the tomato: applications in genetics and breeding. Theor. Appl. Genet. 57:161-170.