HELIA, **22**, Nr. 31, p.p. 95-104, (1999) UDC 633.854.78:632.086.8:632.4

In vitro SCREENING OF SUNFLOWER FOR RESISTANCE TO *Sclerotinia sclerotiorum* (Lib.) de Bary

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Received: June 25, 1999
Accepted: November 15, 1999

SUMMARY

Protoplasts isolated from hypocotyls and leaves of two genotypes of the cultivated sunflower and from stems and leaves of *Helianthus maximiliani* were cultured in the presence of different concentrations of oxalic acid, putative toxin of *Sclerotinia sclerotiorum*. Their viability was determined by staining with fluorescein diacetate during five days. Statistical analysis showed that there was significant difference in reaction to oxalic acid between the genotypes as well as between different organs of the same genotype. Protoplasts of *H. maximiliani* were found to be more resistant to stress with oxalic acid than protoplasts isolated from the two genotypes of the cultivated sunflower which is in accordance with the results obtained in field conditions. Differential reaction of protoplasts isolated from different plant organs to oxalic acid indicates that this method could be used for screening for resistance to different forms of *Sclerotinia*.

Key words: ***Helianthus annuus*, *Helianthus maximiliani*, *Sclerotinia sclerotiorum*, protoplasts, oxalic acid**

INTRODUCTION

White rot caused by the fungus *Sclerotinia sclerotiorum* is the major disease of sunflower (*Helianthus annuus* L.) in countries with the humid climate, while in countries with the moderate climate it causes yield loss in rainy years (Škorić and Rajčan, 1992). This parasite usually attacks all parts of the plant: roots, stalks, leaves, flower buttons and heads (Zimmer and Hoes, 1978). Roots and stem bases can be infected following myceliogenic germination of sclerotia in the spring; flowers and foliage can be infected by ascospores originating from apothecia following

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carpogenic germination of sclerotia later in the year. Although soil solarization can reduce sclerotia numbers in soil (Phillips, 1990), chemical soil sterilization to control sclerotia of this pathogen is impracticable on a field scale, and foliar applications of fungicides to control aerial infections fail to reach lower leaves or stems as the canopy becomes established. There are no suitable cultural control methods (Lumsden, 1979) and no resistant genotypes of cultivated sunflower have yet been found or developed.

The pathogenicity of *Sclerotinia* is complex and is still to be fully explained. However, it is known that cell wall-degrading enzymes, enzymes capable of destroying cellular components and production of oxalic acid, are associated with disease development (Jurgens *et al.*, 1994).

De Bary was the first who associated oxalic acid with *Sclerotinia* infection (Lumsden, 1979). Later, Noyes and Hankock (1981) demonstrated its importance as a factor in the pathogenicity of this fungus, while Hartman *et al.* (1988) found correlation between oxalic acid production and virulence of different isolates of *Sclerotinia*.

There have been several attempts to create a bioassay in which resistance to oxalic acid would be used as an indicator of resistance to *Sclerotinia* (Hartman *et al.*, 1988; Noyes and Hancock, 1981; Raducanu and Soare, 1992; Tu, 1985). In most cases whole plants or their parts were used. Hartman *et al.* (1988) found correlation between growth chamber susceptibility/resistance of tested bean lines to *Sclerotinia* and reaction of the calli of the same lines when grown on medium in which oxalic acid was added. Similar results were obtained by Raducanu and Soare (1992) who studied the effect of oxalic acid on callus initiation on hypocotyl fragments and immature embryos of three sunflower inbreds as well as their weight during culture.

In this paper we describe a method for *in vitro* screening for resistance to *Sclerotinia* by culture of sunflower protoplasts in the presence of oxalic acid.

MATERIAL AND METHODS

Plant material

Two genotypes of the cultivated sunflower and one wild sunflower species were used in the experiment. Seeds of an inbred line of the cultivated sunflower, OD-3389, susceptible to stem attack of *Sclerotinia* (Škorić, personal commucation), and rhizomes of *Helianthus maximiliani* (Schrader) assesion 1631, found to be resistant to white rot (Škorić and Rajčan, 1992), were provided by Institute of Field and Vegetable Crops, Novi Sad, Yugoslavia. Seeds of the commercial sunflower hybrid Emil, susceptible to *Sclerotinia*, were provided by Pioneer Hybrids, Aussonne, France.

Micropropagation of *H. maximiliani*

H. maximiliani was micropropagated by culture of shoot apices and nodal segments on modified MS medium (Murashige and Skoog, 1962) as previously described (Vasić *et al.*, 1998).

Seed germination

Seeds of the two genotypes of the cultivated sunflower were surface sterilized by soaking in 5% solution of calcium hypochlorite for 20 minutes, washed with distilled water and dehulled. Dehulled seeds were sterilized again by dipping in 70% ethanol for one minute followed by soaking in calcium hypochlorite for ten minutes. They were then rinsed three times with sterile distilled water and placed on MS medium with 5 g l^{-1} of sucrose and 8 g l^{-1} of agar, pH 5.8. Seeds were further cultured in the light of intensity of 34 μE m^{-1}, photoperiod 16:8 (light:dark) and temperature 25°C.

Isolation of protoplasts from stems and hypocotyls

Hypocotyls of seven-day-old *in vitro* grown plants of the cultivated sunflower and stems of seven-day-old micropropagated plants of *H. maximiliani* were used for protoplast isolation.

Hypocotyls and stems were cut in half longitudinally and placed in petri dish containing 10 ml of a washing solution (M medium) containing 16 g l^{-1} NaCl, 1.8 g l^{-1} $CaCl_2$ x 2 H_2O, 1 g l^{-1} glucose, 380 mg l^{-1} KCl, 700 mg l^{-1} MES and 80 mg l^{-1} BCP, pH 5.7. After 1.5 hours, the medium was replaced with 9 ml of the same fresh medium and 1 ml of the enzyme mixture composed of 0.75% Caylase M2, 0.525% Caylase 345 and 0.225% Caylase P (Cayla, Toulouse, France). Cell wall digestion was carried out for 13 h in the dark at 26°C. After filtration through 100 mm sieve, protoplasts were purified by floating on 10% Ficoll in the washing medium and rinsed twice in the same medium.

Isolation of protoplasts from leaves

Protoplasts were isolated from leaves of two-week-old plants obtained either by seed germination (cultivated sunflower) or micropropagation (*H. maximiliani*).

Young leaves of *in vitro* cultivated shoots were cut into 2 mm wide strips. The leaf fragments were plasmolysed in 10 ml of M medium for 1.5 hours. After that, M medium was replaced with 9 ml of the same fresh medium and 1 ml of the enzyme mixture composed of 1% cellulase R10, 0.5 % macerozyme R10 (Onozuka, Yakult Housha Co. Ltd, Japan), 0.05 % pectolyase (Seishin Pharmaceutical Co. Ltd, Japan) and 0.01 % driselase (Sigma, St Louis, USA). Digestion was carried out for 20 h in the dark at 26°C. Protoplasts were purified as described above.

Culture of protoplasts

Protoplasts were cultured at the density of 5 x 10^4 per ml in 30 mm petri dishes containing 2 ml of liquid L4M medium (Burrus *et al.*, 1991) in which 10, 1, 0.1, 0.01 and 0 mM of oxalic acid was added (D4, D3, D2, D1 and 0, respectively). Each variant was represented by three petri dishes. Cultures were incubated in the dark at 25°C.

Measurement of protoplast viability

The viability of protoplasts was measured by staining with FDA (Widholm, 1972) which was added to 200 ml samples of protoplasts at a final concentration of 1% and incubated for 5 min. The protoplasts were examined by fluorescence microscopy (Leitz Laborlux 12 microscope, filter I_2). One sample was taken from each petri dish and about 200 protoplasts were examined. The percentage of viability was determined as number of living protoplasts with yellow-green fluorescence per total number of protoplasts counted.

Statistical analysis

For statistical analysis data obtained were expressed as percentage of the mean viability of control (100%). In each variant different petri dishes are treated as one repetition. The Newman-Keuls test was used to separate means ($p<0.05$). Analyses were performed using STATITCF program (ITSF, Paris, France).

RESULTS

Reaction of hypocotyl and stem protoplasts to oxalic acid

The analysis of variance of overall means showed that all three factors (genotype, exposition time, dose) had strong influence on the viability of protoplasts ($p<0.001$). In resistant genotype (*Helianthus maximiliani*) significant difference in viability of stressed comparing to control protoplasts was observed only after five days at the strongest dose of oxalic acid (Table 1). In contrast to this, the decrease of viability of protoplasts of susceptible genotypes was detected as early as one day after beginning of the culture at concentrations higher than 1 mM. The difference in reaction to oxalic acid between resistant and susceptible genotypes was most clearly expressed in the fourth day of culture since presence of oxalic acid reduced viability of Emil and OD-3369 protoplasts at all concentrations while *H. maximiliani* protoplasts did not respond to it (Table 1).

ANOVA of overall means for each day revealed that there was significant difference between tested doses of oxalic acid ($p<0.05$) except in the second and third day where there was no difference between D1 and D2 ($p>0.05$). Further, it showed that the susceptible genotypes did not differ from each other ($p>0.05$) but they differed significantly from *H. maximilani* ($p<0.05$).

Table 1: Viability of hypocotyl and stem protoplasts cultured in the presence of different concentrations of oxalic acid (0=control, D1=0.01 mM, D2=0.1 mM, D3=1 mM, D4=10 mM). Results are expressed as means of three repetitions. The column means followed by different letters differ at $p<0.05$

Genotype	Treatment	Viability (%)				
		1 day	2 days	3 days	4 days	5 days
Helianthus maximiliani	0	100a	100a	100a	100a	100a
	D1	100a	99ab	96ab	99a	100a
	D2	100a	95abc	99a	98a	100a
	D3	100a	95abc	95ab	98a	100a
	D4	100a	96ab	95ab	96a	88b
Emil	0	100a	100a	100a	100a	100a
	D1	99a	89c	95ab	89b	87b
	D2	94a	94abc	90bc	84c	84bc
	D3	85b	80d	76d	78d	79cd
	D4	75c	82d	41f	33f	25g
OD-3369	0	100a	100a	100a	100a	100a
	D1	100a	100a	85c	85c	84bc
	D2	94a	93abc	85c	81cd	77de
	D3	88b	91bc	71d	78d	74e
	D4	76c	73e	53e	50e	37f

Reaction of leaf protoplasts to oxalic acid

Genotype, time of exposition and concentration of oxalic acid had strong influence on the viability of leaf protoplasts ($p<0.001$). Leaf protoplasts of *H. maximiliani* responded more quickly to stress than the ones isolated from stems and there was significant difference in the viability of protoplasts at D4 compared with the control on the second day of culture (Table 2). Similarly to hypocotyl protoplasts, the decrease in viability of leaf protoplasts of the susceptible genotypes was detected as early as one day after the beginning of culture at concentrations higher than 1 mM. The lowest concentration of oxalic acid did not influence the viability of protoplasts of Emil and OD-3369, except on the second day with protoplasts of OD-3369. The difference in reaction to oxalic acid between the resistant and susceptible genotypes was most visible on the second day of culture when the presence of oxalic acid reduced the viability of OD-3369 protoplasts at all concentrations, Emil protoplast had significantly decreased viability at all doses except D1 and *H. maximiliani* protoplasts reacted only to D4 (Table 2).

ANOVA of overall means for each day showed that there were significant differences between all three genotypes, *H. maximiliani* being the first in the rank followed by Emil and OD-3369 ($p<0.05$).

Table 2: Viability of leaf protoplasts cultured in the presence of different concentrations of oxalic acid (0=control, D1=0.01 mM, D2=0.1 mM, D3=1 mM, D4=10 mM). Results are expressed as means of three repetitions. The column means followed by different letter differ at $p<0.05$

Genotype	Treatment	Viability (%)				
		1 day	2 days	3 days	4 days	5 days
Helianthus maximiliani	0	100a	100a	100a	100a	100a
	D1	100a	99a	100a	94abc	100a
	D2	99a	100a	94ab	93abc	99a
	D3	96a	99a	93ab	98ab	97a
	D4	99a	91bcd	99a	90bc	75c
Emil	0	100a	100a	100a	100a	100a
	D1	98a	95ab	96ab	99a	99a
	D2	96a	91bcd	91ab	88c	89b
	D3	86b	86de	72b	79d	79c
	D4	71d	65f	51d	33g	17f
OD-3369	0	100a	100a	100a	100a	100a
	D1	99a	87cde	94ab	93abc	98a
	D2	99a	73e	75b	75d	76c
	D3	78c	58g	62c	59e	68d
	D4	58e	49h	48d	45f	45e

DISCUSSION

In our study, the reaction of protoplast to the challenge with oxalic acid was similar to the reaction of field-grown plants of the same genotypes to the attack of *Sclerotinia*. This is in agreement with the results of Hartman *et al.* (1988) and Raducanu and Soare (1992) who found correlation between tolerance of calli to oxalic acid and resistance of field-grown plants. Further, both genotypes of the cultivated sunflower, i.e., the inbred line (OD-3369) and the hybrid (Emil), reacted to the treatment with oxalic acid.

There were differences in response to oxalic acid between the hypocotyl (stem) and leaf protoplasts of the same genotype. The low concentrations of this compound had little effect on leaf protoplasts of all three tested genotypes, while hypocotyl protoplasts of Emil and OD-3369 were susceptible to them. Furthermore, leaf protoplasts of *H. maximiliani* were more sensitive to oxalic acid than the ones isolated from stems. The difference in reaction of different organs from the same plant to the attack of white rot was also observed in field conditions by Škorić and Rajčan (1992). The authors explained this phenomenon by the fact that the mechanisms of tolerance to the various forms of white rot in sunflowers are controlled by different genetic and other processes (Robert *et al.*, 1987).

A significant drop in protoplast density, caused by protoplast bursting, was observed in the susceptible genotypes at higher concentrations of oxalic acid as early as the second day of culture. Also, in the presence of 10 and 1 mM of oxalic acid, protoplasts of all genotypes failed to divide. Oxalic acid is a strong chelator of calcium and other cations (Lumsden, 1979). It also induces a decrease in pH of infected tissues (Morall *et al.*, 1977). Vallée *et al.* (1995) found that decreased pH of cytoplasm inhibits the division of sunflower protoplasts. Calcium has an important role in the control of mitosis and cell division (Grandin and Charbonneau, 1991) and in the cases when it was chelated the formation of cell plate was inhibited (Jurgens *et al.*, 1994).

In this experiment we studied the reaction of protoplasts to a putative toxin of *Sclerotinia*, i.e., oxalic acid. Beside oxalic acid, other substances such as cell-wall-degrading enzymes and enzymes capable of destroying cellular components are responsible for the development of white mold in natural conditions (Lumsden, 1979). Culture filtrates could contain all these compounds and could thus reproduce better the effect of infection in natural conditions. However, they are also rich in secondary metabolites, growth-inhibiting and -stimulating substances (Yoder, 1980), as well as residues of growth regulators (Gentile *et al.*, 1992). These compounds could affect the protoplast in culture by inhibiting or stimulating their development. Further, the concentration of residues in culture filtrate may vary from culture to culture and have an effect on the reproducibility of results. The utilization of a purified compound such as oxalic acid as toxin eliminates all these constraints.

The advantages of using protoplasts in screening for disease resistance are relatively small quantities of plant material and toxin needed. Further, the bioassays with protoplasts isolated from leaves are non-destructive.

In conclusion, culture of sunflower protoplasts in the presence of oxalic acid was found to be effective for discrimination of resistant and susceptible genotypes. Difference of reaction to oxalic acid of protoplasts from different organs offers the possibility for utilization of this method for screening for resistance to different forms of *Sclerotinia*. This system may also provide a useful tool for studying mechanisms of actions of oxalic acid and response of plants to it contributing in this way to better understanding of the mechanism of attack of *Sclerotinia*.

ACKNOWLEDGEMENTS

The authors wishes to thank Dr. Laurence Deglene for assistance with statistics and helpful discussions.

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INVESTIGACIÓN *in vitro* DE GIRASOL CON RESPECTO A LA RESISTENCIA A *Sclerotinia sclerotiorum* (Lib.) de Bary

RESUMEN

Los protoplastos aislados de hipocotiles y hojas de los genótipos del girasol cultivado y tallos y hojas de la especie *Helianthus maximiliani* eran cultivados en presencia de diversas concentraciones de ácido oxalico, toxina putativa del hongo *Sclerotinia sclerotiorum*. Su variabilidad era determinada por la coloración con diacetato de fluorosceina durante cinco días. El análisis estadistico mostró que existian las diferencias significativas en la relación al ácido oxalico entre los genótipos asi como entre diversos organos vegetales del mismo genótipo. Los protoplastos de *H. maximiliani* han sido constatados como más resistentes al estres de ácido oxalico que esos aislados de dos genótipos del girasol cultivado. Eso es de acuerdo con los resultados obtenidos en las condiciones de campo. La reacción diferente al ácido oxalico determinada en los protoplastos aislados de diversos organos vegetales indica que este metodo puede ser aplicado para probar la resistencia a diversos formas del hongo *Sclerotinia*.

RECHERCHE *in vitro* SUR LA RESISTANCE DU TOURNESOL A LA *Sclerotinia sclerotiorum* (Lib.) de Bary

RÉSUMÉ

Les protoplasmes isolés des hypocotyles et des feuilles de deux génotypes de tournesol de culture et des tiges et des feuilles de l'espèce *Helianthus maximiliani* ont été cultivés en présence de différentes concentrations d'acide oxalique, toxine présumée du *Sclerotinia sclerotiorum*. On a déterminé leur variabilité en les colorant de diacétate fluorescéine pendant cinq jours. L'analyse statistique a montré qu'il y avait des différences importantes dans la réaction à l'acide oxalique autant entre les génotypes qu'entre les différents organes des plantes du même génotype. Il a été confirmé que les protoplasmes de l'espèce *H. maximiliani* étaient plus résistants au stress provoqué par l'acide oxalique que les protoplasmes isolés de deux types de tournesol de culture, ce qui est conforme aux résultats obtenus dans les champs. La réaction différentielle à l'acide oxalique confirmée dans les protoplasmes isolés de différents organes des plantes indique que cette méthode peut être appliquée dans le contrôle de la résistance à différentes formes de *Sclerotinia*.