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Quick method for screening of tolerant sunflower (*Helianthus annuus* L.) genotypes to *Sclerotinia sclerotiorum* at seedling stage

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Abstract. *Sclerotinia sclerotiorum* is a necrotrophic ascomycete fungus with an extremely broad host range. Disease severity is directly correlated with oxalic acid levels in infected tissues. It seems that the most important factor for the resistance of plant cell wall and plasma membrane degradation is resistance against oxalic acid. This research carried out based on completely randomized design with three replications, factors examined in this study included four genotypes of sunflower, six concentrations of oxalate acid and in six different time periods were treated. Positive regression coefficients showed that destruction in all four genotypes increased with increasing concentrations of oxalic acid. This study indicated that cell wall degradation according to the electrolyte leakage, which is fast and accurate method.

Key word: Oxalic acid, Sunflower, cell wall degradation, cluster analysis, *Sclerotinia sclerotiorum.*

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oil crops and due to its high content of unsaturated fatty acids and a lack of cholesterol, the oil benefits from a desirable quality (Nezami et al. 2008, Franchini et al. 2010). *Sclerotinia sclerotiorum* is a necrotrophic fungal pathogen capable of infecting a broad range of hosts, including many economically-significant crops such as sunflower, soybean, and canola (Cessna et al. 2000, Donaldson et al. 2001). *Sclerotinia sclerotiorum* produce millimolar concentrations of oxalate in infected tissues. Oxalate is an essential virulence factor of *S. sclerotiorum* because mutants, which are deficient in oxalate biosynthesis, are less pathogenic than wild-type fungus. In contrast to wild-type fungus, oxalate-deficient *S. sclerotiorum* is unable to produce oxalate during infection of petals, which are an important source of inoculum in the field and during in vitro cultivation (Guimaraes & Stotz 2004). OA secreted by *S. sclerotiorum* is generally believed to help initiate the infection and facilitate disease progression (Bolton et al. 2006). The accumulation of OA has been to shown to be positively correlated with disease severity (Maxwell & Lumsden, 1970).

In addition to cell-wall-degrading activity, OA is known to play a key role in pathogenesis and fungal development (Godoy et al. 1990, Lumsden 1979). OA can degrade or weaken the plant cell wall via acidity or chelation of cell wall Ca^{2+}. Disease severity is directly correlated with OA levels in infected tissues (Noyes et al. 1981, Donaldson et al. 2001). Furthermore, OA plays complex and diverse roles during the infection process, although it is a simple organic substance with limited chemical interactions (Hegedus & Rimmer 2005).

Cell membranes are one of the first targets of many plant stresses. The membrane integrity is altered for the stress; a consequence of this is the increase of the cell permeability which is accompanied by electrolyte leakage from the cell (Ahmadizadeh et al. 2011). Increases in permeability may be caused by pathogen elaborated products or host cell responses to infection. When membrane permeability is drastically altered, cell death follows shortly (Hancock 1972). Electrolyte leakage tests have been widely used to assess the level of plant tolerance to various stresses. These tests determine the degree of cell membrane damage caused by stress based on electrolyte leakage from the cells. The technique is relatively simple, repeatable and rapid and requires inexpensive equipment, can be used on plant material from a variety of cultural systems and it is suitable for the analysis of large numbers of samples (Habibpor et al. 2011, Ahmadizadeh et al. 2011).

The objective was to study the effectiveness and reliability of physiological techniques such as electrolyte leakage tests for screening sunflower genotypes under OA stress and the relation of this trait with the host tolerance and facilitate its introduction within plants farming system prevailing.

Materials and methods

The research carried out in completely randomized design with three replications. Factors examined in this study were 4 sunflower genotypes (Tub, SAT1, SAT2 and CMS-Farox), 6 concentrations of oxalate acid (oxalic acid dehydrate Cat No: 247537; Molecular Weight 126.07 from Sigma Aldrich company) (5, 10, 15, 20, 30 and 40 mM) were treated. Plants were grown in the greenhouse (25°C at 16h light, 8 hr dark photoperiod). The plants in two-cotyledon stage from root were placed in different concentrations of oxalate acid in 6 different time periods (2, 6, 12, 24, 48 and 72 h) were treated. After the elapse of time the leaves cut from the collar were washed by twice distilled water, and electrical conductivity were measured by and EC meters. Data were analyzed by SPSS (vers. 18.0).

Results and Discussion

Factorial analysis for the electrical conductivity (cell wall degradation index) showed significant differences among genotypes which indicate that there was genetic variation in response to oxalate acid (Table 1). Interaction between genotype and time was significant that indicated the genotypes had different response in times of treatment with OA. Also Interaction between the times of treatment with OA and OA

Table 1. Factorial analysis for electrical conductivity.

S.O.V	D.F	Mean Square
		Electrical conductivity
Time (T)	5	9423.3**
Dose (D)	5	2351.97**
Genotype (G)	3	2022.13**
T * D	25	93.28**
T * G	15	167.55**
D * G	15	35.42**
T * D * G	75	37.23**
Error	288	2.299

** = significant at 1% level
T: Time, D: Dose and G: Genotype.

concentration was significant (Table 1). However, this result was not unexpected that genotypes had differences in tolerance to *S. sclerotiorum* disease. ANOVA was performed separately for each concentration. At every levels of oxalic acid (OA) concentration were analyzed for EC. Consider to the result of analysis, significant differences between genotypes were observed (P<0.01). This means that there was variation between genotypes, also between times of treatment with OA was difference significant (Table 2).

The results of the comparing genotypes in term of EC at the 5% level by Duncan's method showed that all dose, the least cell wall degradation occurs in the first 2 hours (Table 3). This was agreement with result of Cessna et al. (2000). At doses of 15 and 20 mM treatments was similar trend of changes over time (Table 3). So to increase the time to 12 hours, the damage was increased, but when the degradation was increased from 24 hours to 72 hours, there was no difference in the rate of degradation. Seems to be the time of maximum membrane degradation occurs 12 hours to 24 hours and then the same process takes degradation. Kim et al. (2008) in their study found that was not significant different between 24 to 40h in term of relative cell death. The result of other researcher demonstrated that oxalate may inhibit a signaling step positioned upstream of oxidase activation into the plant cell cytosol (Cessna et al. 2000). Since the doses of 24, 48 and 72 h, no significant difference in cell membrane damage. Therefore, we could suggest the time of 24 hours for the treatment of OA as proteomics and transcriptional studies. Kim et al. (2008) showed that no significant difference in cell viability was observed in the treatment of K-OX and water until 48 h after treatment was initiated. However, from 48 through 96 h, substantial increases in cell death were observed in oxalate treated leaf disks. By 96 h, extensive cell death and tissue collapse occurred.

Tub genotype at all doses was significantly the least degradation the cell wall (Table 4). Genotypes SAT1, SAT2 and CMS-Farokh as susceptible genotypes were not significant different the amount of cell wall degradation. Regression coefficient was positive and significant of the cell wall degradation at different dose for genotypes (Table 5) showed that destruction in all four genotypes increased with increasing concentrations of OA. In other words, degradation the cell wall in all four genotypes increased with increasing concentrations of OA, then the degradation of the cell wall can be

Table 2. ANOVA for electrical conductivity at different levels of oxalic acid concentration.

S.O.V	D.F	Mean Square					
		5mM	10mM	15mM	20mM	30mM	40mM
Genotype (G)	3	321.9**	391.68**	357.6**	357.56**	1313.2**	1268.4**
Time (T)	5	2155.8**	2073.05**	1509.4**	1509.11**	166.4**	167.3**
G * T	15	0.72 NS	6.75**	1.55 NS	1.58 NS	4.09**	1.5 NS
Error	48	1.59	2.5	2.57	2.75	1.62	3.6
R^2	---	0.99	0.96	0.99	0.98	0.98	0.96

** = significant at 1% level

Table 3. Results of mean comparison of electrical conductivity at different levels of oxalic acid concentrations.

DOSE	5.00 mM	10.00 mM	15.00 mM	20.00 mM	30.00 mM	40.00 Mm
2hr	8.333a	10.916a	15.750a	18.166a	20.916a	22.500a
6hr	10.083b	13.166b	17.416b	22.416b	24.666b	27.666b
12hr	16.833c	19.500c	22.416c	30.500c	35.166c	39.583c
24hr	24.833d	30.666d	35.833d	42.416d	40.416d	43.500d
48hr	28.323e	37.166e	38.333d	42.500d	42.666e	42.000d
72hr	36.500f	42.833f	42.416d	43.583d	43.400e	45.300e

Values with the same superscript letters are non-significantly different at P < 0.05.

Table 4. Results of mean comparison of genotypes at different doses of oxalic acid.

Genotype	5mM	10mM	15mM	20mM	30mM	40mM
Tub	16.444[a]	18.722a	18.722a	26.611a	30.666a	32.944a
sat1	24.388[bc]	28.222b	32.166b	34.888b	36.944b	39.166b
sat2	24.944[bc]	27.666b	32.333b	35.611b	36.333b	38.055bc
cms-farox	25.277[c]	28.222b	31.555b	35.944b	36.888b	39.388c

Values with the same superscript letters are non-significantly different at P < 0.05.

used as an indicator of OA. The oxidative burst, the controlled release of O2 and H2O2, is one of the earliest and most universal responses observed in plants following pathogen challenge (Apel & Hirt 2004, Torres et al. 1998).

Regression analysis demonstrated that was quite different susceptible genotypes (SAT1, SAT2, CMS-Farokh) threshold of their final destruction by an average of 24 hours after OA treatment (EC>40) (Table 5). If the threshold for the resistant genotype, even after 72 hours of treatment oxalic acid did not take place, however, with increasing time of treatment of OA was increased in all genotypes studied. And the differentiation between resistant and susceptible genotypes was observed at 12 h of OA treatment (Figs 1, 2). The regression results for the different genotypes (susceptible and resistant) confirmed this (Table 5).

Table 5. Regression coefficient, concentration of oxalic acid in genotypes.

Genotype	A	B	R^2	R^2 adj
Tub	13.7	0.515	0.92	0.906
SAT1	24.53	0.40	0.90	0.88
SAT2	25.15	0.36	0.84	0.81
CMS-Farox	24.93	0.39	0.89	0.87

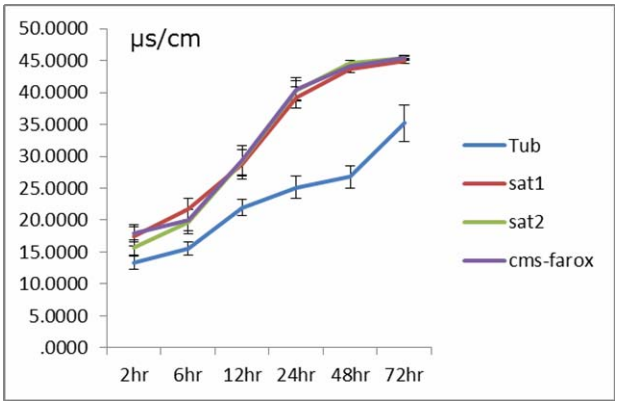
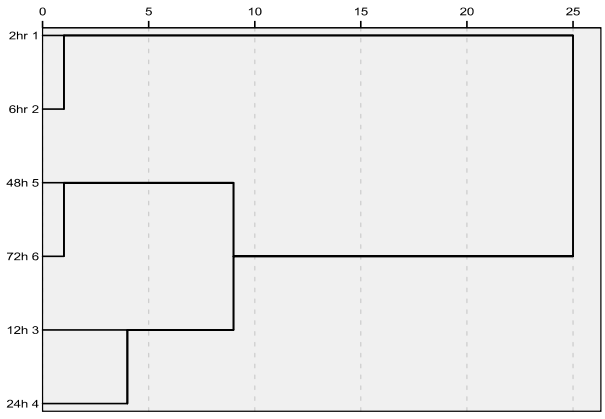


Figure 1. Genotypes deterioration in different times of the treatment of oxalic acid.



Figue 2. Grouping for times of oxalic acid treatment.

Results of cluster analysis for times in term of EC as an index of cell wall degradation showed that 2 and 6 hours of treatment were placed in a group. 12 and 24 hours placed in the second cluster and 48 and 72 hours were in the other cluster (Fig. 2). 2 and 6 hours times and 12 and 14 times had the most distance in grouping, therefor these times were suitable for selected to study the molecular and genetic genotypes (Fig. 2).

Genotypes respond was different to the doses of OA (Fig. 3). So with increasing doses of OA, the cell wall degradation (EC) was increased in all genotypes. 30mM oxalic acid concentration had the most cell wall degradation, but it didn't have significant different with 40mM concentration. Genotypes resistant to cell damage at lower than doses of 15mM were a slower trend, and by increasing the dose of 15 to 20mM its ascending trend and increased EC index. Therefore, the dose of OA concentration for molecular studies was one of the best treatments. The results showed that the grouping of different doses of OA, 2, 6 and 15 mM were placed in one cluster and 20, 30 and 40 mM placed in the second cluster. Thus, 5 mM and 20 mM doses had the most distance in grouping, therefor these doses were suitable for selected to study the molecular and genetic genotypes (Fig. 4). The result indicated that the bulk of the oxalic oxidase activity was localized in the cell wall, which is consistent with the former findings (Wakabayashi et al. 2011). Fungi when infecting host the release of OA, the degradation cell wall (Maxwell & Lumsden 1970) and MAPKK activity is enhanced in an acidic environment this activity can be also degradation the cell wall, causing programed cell death signals (PCD) become active (Kim et al. 2008).

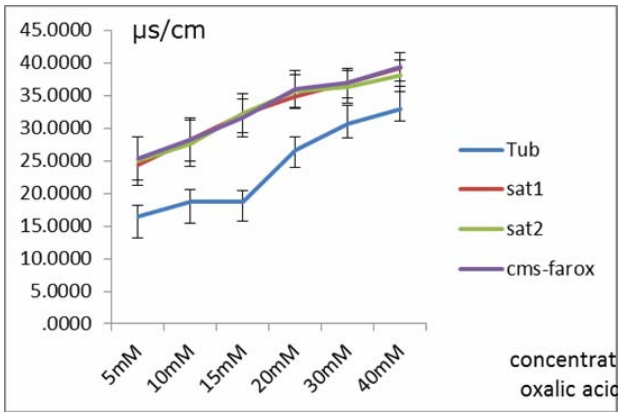


Figure 3. Genotypes deterioration at different levels of oxalic acid concentrations.

Conclusion

For many years, OA has been known as a key determinant in *Sclerotinia* pathogenicity, initially because OA was found in high concentrations (>10 mM) in diseased plant tissue. Accordingly, this correlation led to speculation as to how OA secretion might enhance *Sclerotinia* virulence (Dutton & Evans 1996). In this study we showed that cell wall degradation according to the EC, which is fast and accurate method. Cell wall degradation involves erosion of the cuticle, removal of matrix polysaccharides, and some degradation of cellulose. It appears that the most important factor for the resistance of plant cell wall and plasma membrane degradation is resis-

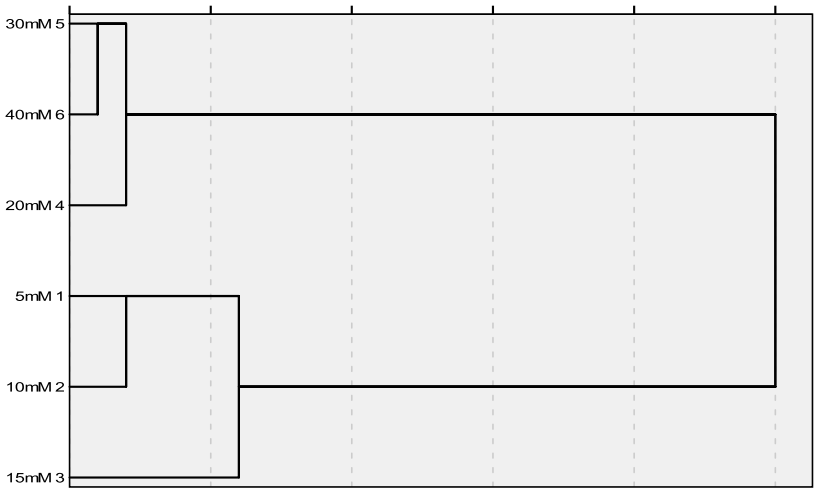


Figure 4. Grouping for levels of oxalic acid concentrations.

tance against oxalic acid. This method can be used for a large number of samples and may be applicable to rapid evaluation of fungal pathogen resistance in large number of genotypes.

References

Ahmadizadeh, M., Valizadeh, M., Zaefizadeh, M., Shahbazi, H. (2011): Antioxidative protection and electrolyte leakage in durum wheat under drought stress condition. Journal of Applied Sciences Research 7(3): 236-246.

Apel, K., Hirt, H. (2004): Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55:373-399.

Bolton, M.D., Thomma, B.P.H.J., Nelson, B.D. (2006): Sclerotinia sclerotiorum (Lib.) de Bary: biology and molecular traits of a cosmopolitan pathogen. Molecular Plant Pathology 7: 1-16.

Cessna, S.G., Sears, V.E., Dickman, M.B., Lowa, P.S. (2000): Oxalic acid, a pathogenicity factor for *sclerotinia sclerotiorum*, suppresses the oxidative burst of the host plant. The Plant Cell 12: 2191–2199.

Donaldson, P.A., Anderson, T., Lane, B.G., Davidson, A.L., Simmonds, D.H. (2001): Soybean plants expressing an active oligomeric oxalate from the wheat *gf-2-8* (germin) gene are resistant to the oxalate-secreting pathogen *sclerotinia sclerotiorum*. Physiological and Molecular Plant Pathology 59: 297-307.

Dutton, M.V., Evans, C.S. (1996): Oxalate production by fungi: Its role in pathogenicity and ecology in the soil environment. Canadian Journal of Microbiology 42: 881-895.

Franchini, M.C., Hernández, L.F., Lindström, L.I. (2010): Cuticle and cuticular wax development in the sunflower (*Helianthus annuus* L.) pericarp grown at the field under a moderate water deficit. International Journal of Experimental Botany 79: 153-161.

Godoy, G., Steadman, J.R., Dickman, M.B., Dam, R. (1990): Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on Phaseolus vulgaris. Physiological and Molecular Plant Pathology 37: 179–191.

Guimaraes, R.L., Stotz, H.U. (2004): Oxalate production by Sclerotinia sclerotiorum deregulates guard cells during infection. Plant Physiology 136: 3703–3711.

Habibpor, M., Valizadeh, M., Shahbazi, H., Ahmadizadeh, M. (2011): Study of drought tolerance with cell membrane stability testing and relation with the drought tolerance indices in genotypes of wheat (*Triticum Aestivum* L.). World Applied Sciences Journal 13(7): 1654-1660.

Hancock, J.G. (1972): Changes in cell membrane permeability in sunflower hypocotyls infected with *sclerotinia sclerotiorum*. Plant Physiology 49: 358-364.

Hegedus, D.D., Rimmer, S.R. (2005): *Sclerotinia sclerotiorum*: when "to be or not to be" a pathogen? FEMS Microbiology Letters 251: 177-184.

Kim, K.S., Min, J.Y., Dickman, M.B. (2008): Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. Molecular Plant-Microbe Interactions 21(5): 605–612.

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

Maxwell, D.P., Lumsden, R.D. (1970): Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. Phyto-pathology 60: 1395–1398.

Nezami, A., Khazaei, H.R., Boroumand Rezazadeh, Z. Hosseini, A. (2008): Effects of drought stress and defoliation on sunflower (*Helianthus annuus*) in controlled conditions. Desert 12: 99-104.

Noyes, R.D., Hancock, J.G. (1981): Role of oxalic acid in *Sclerotinia* wilt of sunflower. Physiological Plant Pathology 18: 123-132.

Torres, M.A., Onouchi, H., Hamada, S., Machida, C., Hammond-Kosack, K.E., Jones, J.D. (1998): Six Arabidopsis thaliana homologues of the human respiratory burst oxidase (gp91phox). The Plant Journal 14: 365-370.

Wakabayashi, K., Soga, K., Hoson, T. (2011): Cell wall oxalate oxidase modifies the ferulate metabolism in cell walls of wheat shoots. Journal of Plant Physiology 168: 1997–2000.