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Relation between clover disease resistance and oxalate-induced changes in protein solubility

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A comparison of response to oxalate was made for two cultivars of red clover (*Trifolium pratense* L.) exhibiting different resistance to *Sclerotinia trifoliorum* Erikss. The cultivar 'Liepsna', which is more sensitive to the pathogen, demonstrated a high sensibility to oxalate solutions in tests with germinating seeds. Conversely, the test of protein solubility showed a slower response of this cultivar to oxalate than did the pathogen-resistant cultivar 'Arimaièiai'. The fast reaction of protein immobilization can be the reason for the resistance of the clover 'Arimaièiai' to pathogenic stress. It is handy to employ oxalate as a selective agent to preselect clover individuals resistant to plant pathogens. This method allows to increase significantly the number of plants resistant to the disease.

Key words: clover, oxalate, plant pathogen, resistance, *Sclerotinia trifoliorum*, *Trifolium pratense*

INTRODUCTION

Red clover (*Trifolium pratense* L.) is a valuable forage legume in Lithuania [10]. Two cultivars, 'Liepsna' and 'Arimaièiai', differently sensitive to clover cancer (*Sclerotinia trifoliorum* Erikss.) were chosen for experiments. The yield of 'Liepsna' varies depending on climatic conditions because of its sensitivity to *S. trifoliorum*. This fungus has a strong killing effect on cv. 'Liepsna'. The solution of this problem is burdened by complicated interactions between the host and the fungus.

The determinants of interaction between the host and the phytopathogen are germin-like proteins [8]. They are involved in the defense against biotic and abiotic stress in plants [12]. Germins are multifunctional proteins, and all theirs functions are associated with the resistance against infection [7], wilt, salt or heat shock. The functions of germin reflect the past evolutionary conditions [3]. The simplest pathogenic biogenic agent is oxalic acid. An 'old' enzyme, oxalate oxidase, and an even 'older' substrate, calcium oxalate, have significant and previously uncontemplated roles in the biochemistry of the extracellular matrix (ECM) of higher plants [5]. Germin, known to be an ECM protein, is an oxalate oxidase. Dissolution of calcium oxalate, and germin-induced degradation of the resulting soluble oxalate, can release Ca^{2+} and H_2O_2, [1] both of which are known to have central roles in the biochemistry of the ECM in higher plants [6]. The glycosylation of germin-like proteins of barley permits them to transit to the ECM structure, affects their solubility from this matrix [6, 11]. Germin also shows superoxide dismutase activity, suggesting the defense against extracellular superoxide radicals to be its important additional role [13]. The findings about oxalate as a source of H_2O_2 are a complement to the contemporaneous advocacy of a central role for H_2O_2 in the signaling processes of higher plants [7]. On the other side, oxalate serves as a signaling agent for plant cells about the beginning of attack [9]. It allows the plant to prepare, through the mobilization and redistribution of resources, for the oncoming attack of phytopathogenic microorganisms [2]. The phytopathogenic fungus *S. trifoliorum* Erikss. excretes into cultural media substances, oxalic acid among them, which exert a complex effect on clover [4]. Our attention was concentrated on the modification of solubility of clover protein as a response to treatment with oxalate. The rate of response to oxalate may reflect the common resistance of a plant to infection. The investigation would enable to prepare methods for selection of clover cultivars resistant to a good many of phytopathogens that excrete oxalic acid.

MATERIALS AND METHODS

The pathogenic fungus *Sclerotinia trifoliorum* Erikss. was obtained from Dr. B. Grigaliûnaitë (Institute of Botany, Vilnius). The red clover *T. pratense* L. cultivars 'Liepsna' and 'Arimaièiai' were obtained from Dr. Habil. A. Svirskis (Lithuanian Institute of Agriculture, Dotnuva).

Electrophoresis of soluble protein. Clover seedlings (about three days old) were over poured with 50–100 mM oxalate solutions for two hours, and then roots were separated. Samples without oxalate served as control. Clover proteins were extracted from 3-d roots with a buffer containing 0.0625M TRIS-HCl, 5% 2-mercaptoethanol, 15% glycerol, 2% SDS, pH 6.8. The crude extracts were centrifuged for 20–30 min at 7000–7500 rpm and heated for 5 min in boiling water. Samples of extracted protein (20–35 μl) were subjected to electrophoresis in discontinuous 7–17% linear gradient polyacrylamide gel slabs in the presence of SDS. The gels were stained with 0.1% Coomassie Brilliant Blue R250 in 50% ethanol, 10% acetic acid. The electrophoregrams were scanned with Mustek-1200 UB Plus at a resolution of 600 dpi.

Photometry of protein. The method for determination of protein concentration is absorption photometry based on Lambert–Beer's law (Equation 1). This equation relates light transmittance (T), substance concentration (c), layer thickness (d) and molecular extinction (ε):

$$T = \exp(-\varepsilon \cdot c \cdot d). \quad (1)$$

Transmittance is a nonlinear function of the concentration. Optical density (OD) is used instead of protein real concentration because of a linear (Equation 2) interrelationship among c, d and OD.

$$OD = -\log(T) = \varepsilon \cdot c \cdot d. \quad (2)$$

The extinction alternates are subject to the dye and the operational wavelength.

Determination of resistance. For determination of clover resistance to oxalate, sterilized seeds were spread out and poured over with oxalate solution and were aseptically germinated on the filtering paper in Petri dishes. Seedlings were grow with 0–250 mM oxalate solutions (pH 4.5) in the dark for 20 h and then washed with 0.5% $KHCO_3$, twice washed with water, and following 20 h the length of roots was measured. Roots < 1.5 mm long were counted as damaged. For every repeat about 350 plants were counted. Every point for 'Arimaièiai' is an average of two repeats and for 'Liepsna' of five repeats. The toxic effects of oxalate solution were counted as the percentage of damaged plants.

For determination of clover resistance to *S. trifoliorum*, the fungus was cultivated in a medium made on the basis of the medium described in [4] and modified (to activate the fungus metabolism) by replacing yeast extract with the extract from clover seedlings. The medium was supplemented with 1 g arabinose, 12 g sucrose and 5 g sodium citrate. Before use, mycelium was slightly crushed. Seedlings (four days old) were poured over by fungal suspension, and after three days the survived and deceased plants were counted.

The statistical analysis of the results was made with the aid of MS Excel 2002 Statistical Analysis Tool Pack.

RESULTS AND DISCUSSION

Our purpose was to prepare a rapid method of preselection of plants resistant to *S. trifoliorum* on the base of new findings about the dominance of oxalate in the phytopathogenic process. Selection of pathogen-proof clover forms is complicated by a multifactorial interaction between the plant and the fungus and by a quick accommodation of this phytopathogen to new cultivars. The toxic effect of oxalate solutions on clover seedlings was studied because of presence of oxalic acid almost in all phytopathogenic processes. The toxic effect of oxalate on these two differently resistant clover cultivars is presented in Fig. 1. It seems that both the cultivars 'Arimaièiai' and 'Liepsna' are composed of two parts differently resistant to oxalate. The sensitive part of clovers dies at 5–7 mM oxalate, and the resistant part survives a 75–100 mM oxalate concentration. The shape of the curves demonstrates that in 'Liepsna' about 65% of plants are sensitive to oxalate and the rest are resistant, whereas in 'Arimaièiai' there is only about 15% of sensitive plants. Such diffe-

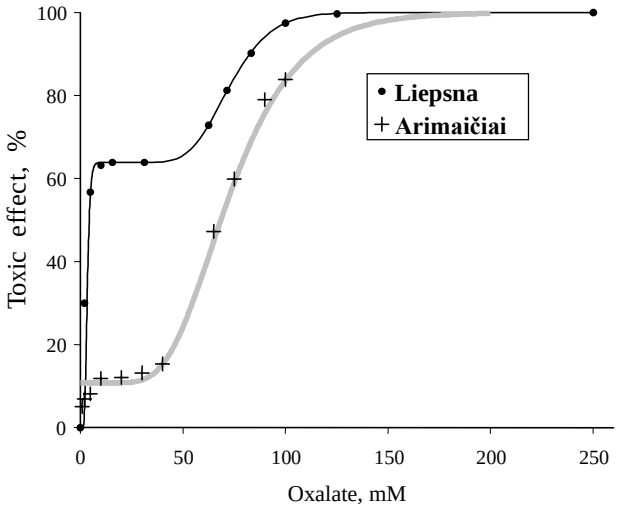

Fig. 1. Toxic effect of oxalate solutions on differently disease-resistant clover cultivars. Each point on the curve represents an average of five repeats (5 × 400 plants) of nonresistant clover 'Liepsna' and two repeats (2 × 350 plants) of resistant 'Arimaièiai'. Toxic effect is presented as percentage of deceased plants. Smooth curves are approximations of experimental data by least square regression method

rences in the percentage content of these two cultivars are enough for the reasoning of the distinct resistance against fungus *S. trifoliorum*.

Examination of protein dissolubility dynamics with the aid of electrophoresis pattern (Fig. 2) demonstrates a considerable loss of protein solubility in the germinating resistant clover 'Arimaièiai' under treatment with oxalate. In all protein zones of disease-resistant 'Arimaièiai' the decrease protein solubility under treatment with oxalate is much more intensive than that of 'Liepsna'. An especially significant reduction of solubility was observed in the peak of heavy protein fractions with 54 kDa molecular weight (Mw) (kilodaltones). The area of the mentioned peak in 'Arimaièiai' under treatment with 50 mM oxalate decreases to 46% in comparison to control (without oxalic acid). Practically no significant changes of protein solubility in disease-sensitive 'Liepsna' have been observed under the treatment. 'Liepsna' showed a reduction in solubility under treatment only in protein zones at Mw~54.5 kDa and Mw~88 kDa. It seems that the immunity of 'Arimaièiai' to *S. trifoliorum* is determined by the ability of clover to produce a rapid response to the alarm signal: to oxalate or maybe to peroxide [13] produced by oxalate-oxidase. As a result of the response, glycosylation of clover proteins permits them to transit to the EMC and affects their solubility from this matrix [7].

A comparison of experimental results on the treatment of clover with a suspension of the pathogenic fungus *S. trifoliorum* Erikss. (Table) and with oxalate solutions demonstrates a good accord between clover resistance to oxalate and to plant pathogens. The effect of the fungus *S. trifoliorum* is equivalent by toxicity to about 20–25 mM of oxalate solution: the toxic (killing) effect of fungal suspension was 63.8 ± 4.4 and the effect of oxalate solution was 63.9 ± 1.7 for cv. 'Liepsna'. 10.8 ± 3.1 and 13.7 ± 2.6 for cv. 'Arimaièiai'.

Table. **Comparison of disease-sensitive 'Liepsna' and resistant 'Arimaièiai' clover cultivars**

Test	Toxic effect to	
	'Liepsna'	'Arimaièiai'
Control	0.5 ± 0.5	3.1 ± 1.7
25 mM oxalate	63.9 ± 1.7	13.7 ± 2.6
50 mM oxalate	65.2 ± 1.5	20.8 ± 1.8
100 mM oxalate	97.4 ± 0.5	83.8 ± 7.4
S. trifoliorum	63.8 ± 4.4	10.8 ± 3.1

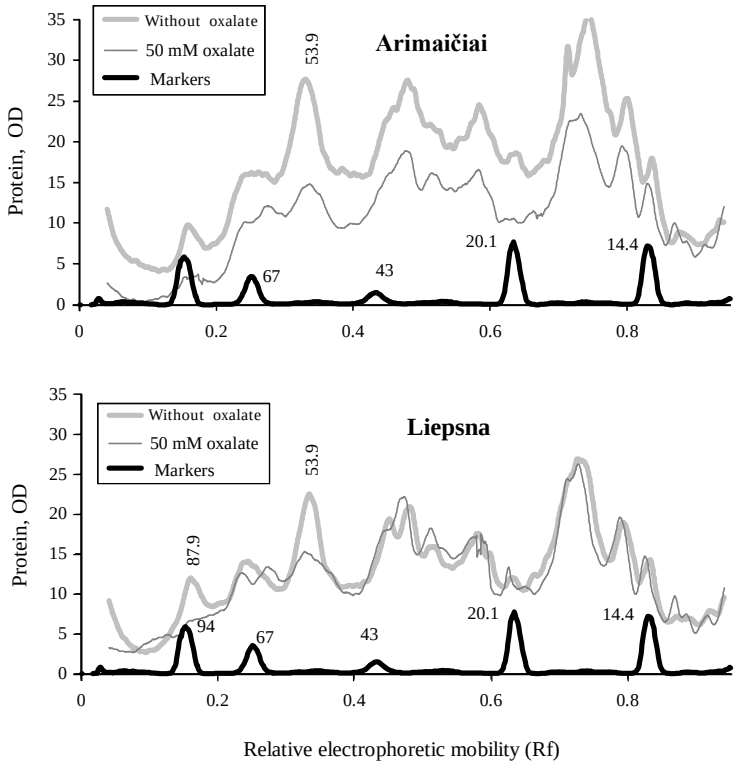

Fig. 2. Oxalate-induced changes of protein solubility of differently disease-resistant clover cultivars. Data on resistant clover 'Arimaièiai' and nonresistant 'Liepsna' are shown. Molecular weights of standard markers and some protein peaks are pointed in kDa

The defense mechanism of clover resistance is not yet clear. Is it related to synthesis of the new defense proteins (processes induced by oxalic acid or by peroxide generated from oxalic acid) or is it permanent? Success in getting an argument in favour of the presence of adaptive processes of the defensive mechanism of a plant would imply the preference of the inducible mechanism. This would offer a prospect of the artificial control of these processes by genetic manipulations. During these manipulations the plants may lose fecundity in the case of the permanence of the defending system, and for this reason the inducible defensive mechanism is preferable over permanent.

CONCLUSIONS

In *Trifolium pratense* plants there are processes controlling the metabolism of oxalic acid and producing signals for plant systems that prevent biotic (and abiotic) stress. The results presented here suggest that the resistance of plants is predetermined by the capacity of clover to respond to oxalate and to produce a stress signal. The rate of protein precipitation onto

EMC, as a result of the response to this signal, can predestinate differences between clover cultivars. The general axis of the interaction between clover and the pathogen *S. trifoliorum* is oxalate. This motivates the use of oxalate as a selective agent to preselect individual plants resistant to *Sclerotinia trifoliorum* Erikss. The first indicator of the immunity of species to the plant pathogen can be a rapid loss of protein solubility under the influence of oxalic acid.

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References

1. Berna A., Bernier F. Regulation by biotic and abiotic stress of a wheat germin gene encoding oxalate oxidase, a H_2O_2-producing enzyme // Journal of Plant Molecular Biology. 1999. Vol. 39(3). P. 539–549.
2. Carter C. and Thornbur, R.W. Tobacco nectarin I. Purification and characterization as a germin-like, manganese superoxide dismutase implicated in the defense of floral reproductive tissues // Journal of Biological Chemistry. 2000. Vol. 275(47). P. 36726–36733.
3. Dunwell J. M., Khuri S. and Gane P. J. Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily // Microbiology and Molecular Biology Reviews. 2000. Vol. 64(1). P. 153–179.
4. Janèys Z. Influence of calcium and magnesium on the interaction between *Sclerotinia trifoliorum* Erikss. and red clover *Trifolium pratense* L. // Biologija. 1995. Vol. 3–4. P. 140–142.
5. Khuri S., Bakker F. T. and Dunwell J. M. Phylogeny, function, and evolution of the cupins, a structurally conserved, functionally diverse superfamily of proteins // Molecular Biology and Evolution. 2001. Vol. 18(4). P. 593–605.
6. Lane B. G. Oxalate oxidases and differentiating surface structure in wheat: germins // Biochemistry Journal. 2000. Vol. 349(Pt 1). P. 309–321.
7. Lane B. G. Oxalate, germins, and higher-plant pathogens // IUBMB Life. 2002. Vol. 53(2). P. 67–75.
8. Membre N., Bernier F., Staiger D. and Berna A. Arabidopsis thaliana germin-like proteins: common and specific features point to a variety of functions // Planta. 2000. Vol. 211(3). P. 345–354.
9. Schweizer P., Christoffel A. and Dudler R. Transient expression of members of the germin-like gene family in epidermal cells of wheat confers disease resistance // Plant Journal. 1999. Vol. 20(5). P. 541–552.
10. Svirskis A. Yield increasing of herbage legume species by plant breeding. Habil D. thesis. Dotnuva, 1995. 156 p.
11. Vallelian-Bindschedler L., Mosinger E., Metraux J. P. and Schweizer P. Structure, expression and localization of a germin-like protein in barley (*Hordeum vulgare* L.) that is insolubilized in stressed leaves // Journal of Plant Molecular Biology. 1998. Vol. 37(2). P. 297–308.
12. Wojtaszek P., Piclewska M., Bolwell G. P. and Stobiecki M. Secretion of stress-related proteins by suspension-cultured *Lupinus albus* cells // Acta Biochimica Polonica. 1998. Vol. 45(1). P. 281–285.
13. Woo E. J., Dunwell J. M., Goodenough P. W., Marvier A. C. and Pickersgill R. W. Germin is a manganese containing homohexamer with oxalate oxidase and superoxide dismutase activities // Nature Structural Biology. 2000. Vol. 7(11). P. 1036–1040.

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DOBILO ATSPARUMO LIGOMS PALYGINIMAS SU JO BALTYMØ TIRPUMO KITIMU VEIKIANT OKSALATU

S a n t r a u k a

Tiriama atsparios ir neatsparios fitopatogenui *Sclerotinia trifoliorum* Erikss. raudonøjø dobilø linijos reakcija á oksalatà. Daiginimo oksalato tirpaluose testas parodë, kad neatspari fitopatogenui dobilø 'Liepsna' linija buvo jautresnë ir oksalatui. Testuojant pagal baltymø ekstrahuojamumo kitimà, ði linija pasiþymëjo pavëluota reakcija á oksalatà, palyginus su atspariàja – 'Arimaièiai' linija. Augalø gebëjimas greitai imobilizuoti tirpius baltymus gali bûti vienas ið patikimø testø, patogus naudoti atrenkant augalus, atsparius fitopatogeniniams organizmams.

Raktaþodþiai: dobilas, oksalatas, fitopatogenas, atsparumas, *Sclerotinia trifoliorum, Trifolium pratense*

Зенонас Янчис, Регина Вишняускене

СРАВНЕНИЕ БОЛЕЗНЕУСТОЙЧИВОСТИ КЛЕВЕРА С ИЗМЕНЕНИЕМ РАСТВОРИМОСТИ ЕГО БЕЛКОВ ПОД ВОЗДЕЙСТВИЕМ ОКСАЛАТА

Р е з ю м е

Исследованы и сравнены различные ответные реакции на воздействие растворами щавелевой кислоты, стойкой к фитопатогену *Sclerotinia trifoliorum* Erikss. 'Аримайчай' и чувствительной – 'Лепсна', сортов клевера. Стойкий сорт отличался более ярко выраженной реакцией по потере растворимости белков при воздействии оксалатом. Эта отличительная черта может быть использована на ранних этапах селекции устойчивых к фитопатогенам растений.

Ключевые слова: клевер, оксалат, фитопатоген, устойчивость, *Sclerotinia trifoliorum, Trifolium pratense*