Imitation of phytopathogenic stress with oxalate in red clover

Z. Jančys,
R. Vyšniauskienė

Institute of Botany,
Žaliųjų ežerų 49,
LT-2021, Vilnius, Lithuania,
E-mail: zenonas@botanika.lt

The red clover cultivars resistant and susceptible to *Sclerotinia trifoliorum* Erikss. were compared by their response to oxalate. More susceptible cultivars demonstrated an intense response to oxalate solutions in tests with seed germination or rate root growth. The test of protein solubility showed that less resistant plants had a slower response to oxalate. Fast immobilization of proteins can be the cause of clover resistance to pathogens. It is expedient to employ oxalates as a selective agent to discriminate clover individuals resistant to plant pathogens.

Key words: plant pathogen, oxalate, resistance, clover

INTRODUCTION

Red clover (*Trifolium pratense* L.) is a widespread and valuable forage grass in Lithuania [1]. The cultivars 'Arimaičiai' (registered 1996 in Lithuania) and 'Liepsna' hawe been chosen for experiments because of their economic importance. The yield of 'Liepsna' varies depending on climatic conditions. Particularly, 'Liepsna' is very sensitive to *Sclerotinia trifoliorum* Erikss. It is evident that the determinants in plant pathogenesis are germin-like proteins. They are involved in the defense against biotic and abiotic stress in plants. Germins are multifunctional proteins, and all their functions are associated with resistance to infection, frost, wilt, salt or heat shock. The functions of germins reflect the past evolutionary conditions. The most primitive 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. 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, both of which are known to have central roles in the biochemistry of the ECM in higher plants [2]. Germin is also shown to have superoxide dismutase activity suggesting that the defense against extracellular superoxide radicals is an important additional function for germins [3]. Findings about oxalates 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 [4]. On the other side, oxalates may serve as a signaling agent for a plant about the beginning of attack. It allows the plant to prepare, through the mobilization and redistribution resources, against oncoming attack by phytopathogenic microorganisms. The rate of response to infection maybe reflects the complex resistance of plants. The glycosylation of germin-like proteins of barley permits them to pass to the ECM structure, affects their solubility from this matrix [5]. The phytopathogenic fungus *S. trifoliorum* Erikss. excretes into cultural media substances, oxalic acid among them, that exert a complex effect on clover [6]. Our regard was concentrated on the modification of clover protein solubility in connection with oxalic acid. It would enable to prognosticate and to preselect clover cultivars or individuals resistant to phytopathogenic oxalate producers.

MATERIALS AND METHODS

Red clover *T. pratense* L., cultivars 'Liepsna', and 'Arimaičiai' were obtained from the Dr. Habil. A. Svirskis (Lithuanian Institute of Agriculture, Dotnuva). With the purpose to evaluate the weight of the resistance to oxalates in common immunity, we selected from cv. 'Liepsna' (at 100 mM oxalate 1% survival) the strain O×90.3 resistant to oxalate. The pathogenic fungus *Sclerotinia trifoliorum* Erikss. was obtained from Dr. B. Grigaliūnaitė (Institute of Botany, Vilnius).

Electrophoresis of soluble clover proteins. Seedlings (about three days old) were overpoured with 50–100 mM oxalate solutions for 2 h and then the roots were separated. Samples without oxalate served as control. Clover proteins were extracted from

three-day-old roots with a sample buffer (ratio 1:1) 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 proteins (25–35 µl) were subjected to electrophoresis in 10% polyacrylamide gel slabs in the presence of SDS [7]. These gels were stained with 0.1% Coomassie Brillant Blue R250 in 50% ethanol, 10% acetic acid. Electrophoregrams were scanned with a Mustek-1200 at a resolution of 600 dpi.

Photometry: transformation of transmittance to concentration. The method of determination of protein concentration is therefore absorption photometry based on the Lambert–Beer law (Equation 1). This equation correlates 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 with the concentration. Optical density (OD) is usable instead of protein's real concentration because of a linear relationship between c, d and OD (Equation 2):

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

Extinctions are alternately subjected to the dye. The purpose of higher precision needs employment of full information from the color scan. Every pixel presents transmittance at three colors: red, green, blue (RGB). The common OD is a weighed average of respective densities (Equation 3):

$$OD = c \cdot d \cdot (\varepsilon_r \cdot W_r + \varepsilon_g \cdot W_g + \varepsilon_b \cdot W_b) / (W_r + W_g + W_b). \quad (3)$$

Concrete parameters of extinction can easily be found by least square regression methods. Weights (W) of each color are taken as a function of transmittance and calculated by our empiric formula (Equation 4):

$$W_{rgb} = (T_{rgb} \cdot N \cdot (2 - T_{rgb} \cdot N))^2. \quad (4)$$

N is the normalizing coefficient (0.00784) for transforming the meaning of pixel (0–255) into transmittance units.

Determination of plant resistance. The pathogen was cultivated in the medium made on the basis of the media described in [8, 9] and modified (for fungus metabolism activation) by replacing yeast extract with extract from clover seedlings. The media was supplemented with 1 g arabinose, 12 g sucrose and 5 g sodium citrate (for augmenting the buffer capacity). Oxalate production reached 30–40 mM in the medium containing 5 g/l sodium citrate, and the toxicity of the solution to clover reached maximum. Just before use the mycelium was slightly crushed. Seedlings (4–5-day-old) were poured over by fungal suspension, and after 3 days the survived plants were counted.

For determination of clover resistance to oxalate, the seeds were sterilized for about 9–10 min by concentrated sulphuric acid and rinsed 5 times in sterile water. Sterile seeds were spread out and poured over with oxalate solution and were aseptically germinated on the filtering paper in Petri dishes. Seedlings were growing with 0–250 mM oxalate solutions in the dark for 20 h, then were twice washed and after another 24 h the root length was measured. Roots <1.5 mm long were counted as not resistant (deceased). Every point for 'Liepsna' an average of five repeats, for 'O×90.3' of two or three repeats and for 'Arimaičiai' of one or two repeats. For every repeat 350–400 plants were counted. The statistical treatment of the experimental results was made with the aid of MS Excel 2002 Statistical Analysis Tool Pack and Statistica 5.5 software.

RESULTS AND DISCUSSION

Selection of decease-proof clover forms is complicated by a multifactorial interaction between the plant and the fungus and by a quick adaptation of this plant pathogen to new cultivars. The object we propose to self to have a rapid method for preselection of resistant plants. Electrophoresis pattern of the soluble proteins demonstrates decline of solubility of proteins in germinated resistant clover 'Arimaičiai' (Fig. 1). An especially conspicuous reducing of solubility was observed in the heavy fractions, whereas the sensitive clover cultivar 'Liepsna' showed no significant difference, except protein peaks with a relative electroforetic mobility (R_f) 0.165 (p1) and R_f 0.334 (p2) (molecular weight 87.95 kDa and 54.3 kDa, respectively). The peak under treatment with 50 mM oxalate decreased to 57.36 ± 2.42%, and the second peak p2 decreased to 66.86 ± 1.31% *versus* control. A wide zone between two markers (20.1 kDa and 43 kDa) was taken for comparison. Under treatment with oxalate, no significant changes (103.01 ± 12.05%) in this zone were observed in the 'sensitive 'Liepsna', meanwhile the resistant cv. 'Arimaičiai" decreased the solubility of proteins under treatment with oxalate much more intensively. The peak p1 decreased to 35.95 ± 3.62% and the peak p2 to 54.15 ± 1.86%. The solubility of proteins decreased to 67.49 ± 5.6% in the wide zone

mentioned above, there 'Liepsna' showed no changes. The immunity of 'Arimaičiai' to *S. trifoliorum* seems to be determined by the ability of the clover to respond rapidly to the alert signal – oxalate (or maybe to peroxide [2, 4] produced by oxalate-oxidase). The resulting glycosylation of clover proteins (and jointly germins) permits them to pass to the extracellular matrix structure, affects their solubility from this matrix [5].

Table. **Clover cultivar survival and the toxic effect of oxalate**

Cultivars	Toxic effect of oxalate, %			Survival with *S. trifoliorum*, %
	Control	50 mM	100 mM	
Liepsna	0.0	63.88 ± 0.7	97.43 ± 0.5	36.17 ± 0.44
Arimaičiai	3.1 ± 1.7	13.69 ± 2.6	83.83 ± 7.4	89.17 ± 6.10
O × 90.3	0.0	0.98 ± 0.5	51.5 ± 2.1	94.33 ± 1.86

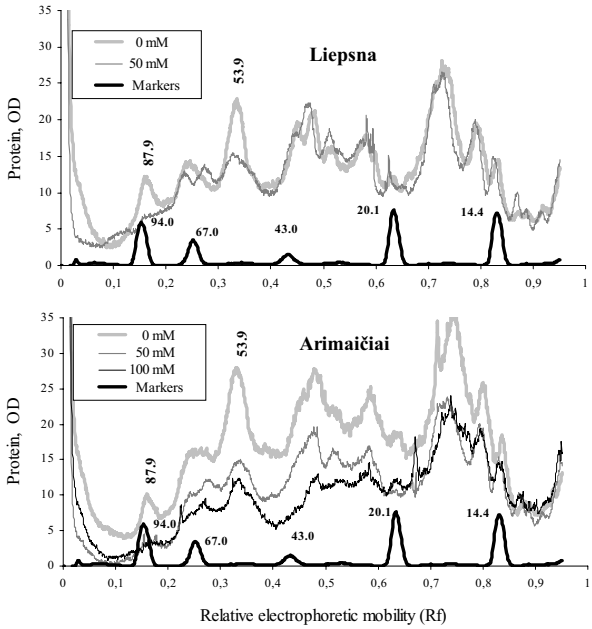

Fig. 1. Stress-induced changes in protein solubility as a response to oxalate. The are exposed sensitive to *S.trifoliorum* Erikss. cv. 'Liepsna' and resistant cv. 'Arimaičiai'. Molecular weights (Mw) of standard markers are pointed in kilodaltones (horizontally). Molecular weight of two peaks p1 and p2 is pointed over them (vertically)

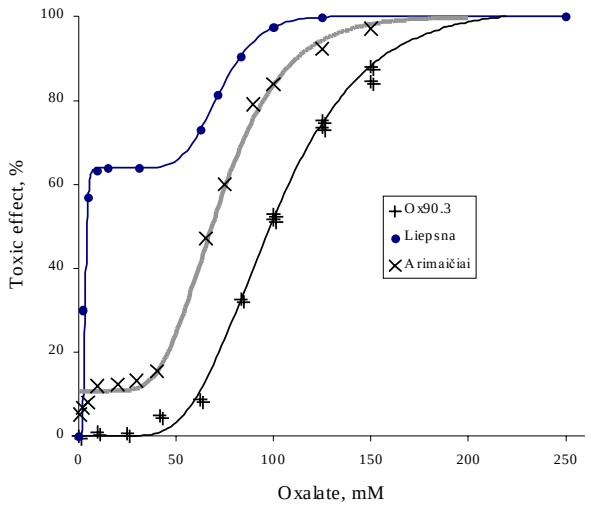

Fig. 2. Resistance of clover cultivars to toxic solutions of oxalate. Curves were solved by least square regression method

Figure 2 shows the toxic effect of oxalic acid on these two clover cultivars and one strain resistant to oxalate. The shape of the curve clearly demonstrates that cultivar 'Liepsna' is composed of two parts differing in the resistance to oxalate: about 65% is sensitive and the remainder is resistant, whereas 'Arimaičiai has only about 15% of sensitive plants. The strain O×90.3 has no significant admixture of oxalate-sensitive plants. A comparison of experimental results obtained with suspension of *S. trifoliorum* Erikss. (Table) and with pure oxalate revealed a correlation between clover resistance to the pathogen and cultivar resistance to oxalate. It should be noted that survival with the pathogen much more fulfils conditions at 50 mM oxalate than at 100 mM. Apparently, this concentration is close to the natural levels in soil.

Our results suggest that the plants of *T. pratense* L. selected by their resistivity to oxalate showed a higher resistance to *S. trifoliorum* Erikss. That confirms yet again our presumption that the general axis of the interaction between *S. trifoliorum* and clover is a complex of genes responsible for the metabolism of oxalic acid. This impels to use oxalate as a selective agent to individual plants resistant to *S. trifoliorum*. The first indicator of the immunity species to plant-pathogens must be speedy immobilization of soluble proteins as a response to oxalic acid.

References

1. Svirskis A. Yield increasing of herbage legume species by plant breeding. Thesis of the dissert for habil dr Lithuanian Inst of Agriculture (Dotnuva) 1995.
2. Lane BG. Biochem J 2000; 349(Pt 1): 309–21.
3. Woo EJ, Dunwell JM, Goodenough PW, Marvier AC, Pickersgill RW. Nat Struct Biol 2000; 7(11): 1036–40.
4. Lane BG. FASEB J 1994; 8(3): 294–301.
5. Domon JM, Dumas B, Laine E, Meyer Y, David A, David H. Plant Physiol 1995; 108(1): 141–8.
6. Jančys Z. Biologija 1995; 3–4: 140–2.
7. Laemmli UK. Nature 1970; 227: 680–5.
8. Maxwel DP, Lumsden RD. Phytopathology 1970; 60: 1395–8.
9. Punja ZK, Jenkins SF. Mycologia 1984; 76(5): 947–50.

Z. Jančys, R. Vyšniauskienė

RAUDONOJO DOBILO FITOPATOGENINIO STRESO IMITACIJA OKSALATU

S a n t r a u k a

Lygintos atsparios ir neatsparios *Sclerotinia trifoliorum* Erikss. fitopatogenui raudonųjų dobilų linijos pagal jų reakciją į oksalatą. Testuota pagal šaknelių augimo sulėtėjimą. Rasta, kad jautresnės fitopatogenui dobilų linijos ryškiau reaguoja į oksalato tirpalą. Tiriant tirpių baltymų kiekio kaitą, pažymėtina, kad jautrių dobilų linijų reakcija buvo lėtesnė negu atsparių. Augalų gebėjimas greitai imobilizuoti tirpius baltymus gali būti viena iš priežasčių, lemiančių linijos atsparumą fitopatogenui. Atsparumą oksalatui patogu naudoti kaip testą atrenkant fitopatogenams atsparius augalus.